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Docket No.: HO-P03236US0
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Makrina D. Savvidou *et al.*
Application No.: 10/553,462
Filed: April 17, 2004
For: SCREEN FOR PRE-ECLAMPSIA

Confirmation No.: 8934
Art Unit: 1632
Examiner: A. K. Singh

APPEAL BRIEF

TABLE OF CONTENTS

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APPEAL BRIEF

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Commissioner:

Appellants hereby submit this Appeal Brief to the Board of Patent Appeals and Interferences in response to the Final Office Action dated December 9, 2010 ("the Action"); the Notice of Appeal and Pre-Brief Conference Request that were filed June 8, 2011, and the Examiner Interview Summary Record of July 14, 2011. A Petition for Extension of Time of One Month and the requisite fee are filed herewith.

The fee for filing this Appeal Brief is submitted herewith. The Commissioner is hereby authorized to deduct any underpayment of fees or any additional fees required under 37 C.F.R. §§ 1.16 to 1.21 in connection with the filing of this paper from Fulbright & Jaworski L.L.P. Account No.: 06-2375/HO-P03236US0.

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, University College London.

II. RELATED APPEALS AND INTERFERENCES

None.

III. STATUS OF THE CLAIMS

Claims 1, 6, 7, 8, and 11 are on appeal. Claims 2-5, 9-10, and 12-28 are canceled.

IV. STATUS OF AMENDMENTS

There are no current amendments.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claim 1 requires a method of determining that a pregnant woman is at risk of developing pre-eclampsia or that her fetus is at risk of developing intrauterine growth restriction (IUGR) (page 1, lines 30-32). The method comprises (a) measuring asymmetric dimethylarginine (ADMA) in a plasma sample (page 3, line 10) taken from a pregnant woman at a stage of pregnancy from 23 to 25 weeks gestation (page 3, line 33- page 4, line 1); and (b) determining that the woman is at risk of developing pre-eclampsia or her fetus is at risk of developing IUGR if the level of ADMA in the plasma sample is greater than 1.5 μmol/L (page 4, lines 26-29) (page 1, line 30- page 2, line 3).

As claimed in claim 6, in some embodiments, the method includes determining that the woman is at risk of developing pre-eclampsia or determining that her fetus is at risk of developing IUGR comprises determining that the woman's ADMA level is at least 3 times the normal pregnancy level. (page 4, lines 30-31)

As claimed in claim 7, in some embodiments the determining that the woman is at risk of developing pre-eclampsia or determining that her fetus is at risk of developing IUGR comprises determining that the woman has an increase in the ADMA/symmetric dimethylarginine (ADMA/SDMA) ratio that is greater than the normal pregnancy ratio. (page 5, lines 11-14)

As claimed in claim 8, in some embodiments the method comprises determining that the ADMA/SDMA ratio is at least 5 times more than the normal pregnancy ratio. (page 5, lines 22-23)

As claimed in claim 11, in some embodiments the method further comprises carrying out Doppler waveform analysis of the uterine arteries and/or flow-mediated dilatation of the brachial artery in the woman. (page 7, lines 13-16)

VI. GROUND OF REJECTION TO BE REVIEWED ON APPEAL

Whether claims 1 and 6-8 are unpatentable under 35 U.S.C. §103(a) over Holden *et al.* (*Am J Obstet Gynecol.* 1998; 178(3):551-6), Ellis *et al.* (*Acta Obstr. Gynecol Scand* 2001,80,602-608) and Boger (WO 2002114873, 2/21/2002).

Whether claims 1 and 11 are unpatentable under 35 U.S.C. § 103(a) as being unpatentable over Holden, Ellis and Boger as applied to claims 1 and 6-8, and further in view of Albaiges *et al.* (*Obstet Gynecol* 2000;96:559-64)

VII. ARGUMENT

A. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that

findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an Examiner's position on appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Rejection under 35 USC § 103(a)

1. Claims 1 and 6-8

Claims 1 and 6-8 were rejected under 35 U.S.C. §103(a) over Holden *et al.* (*Am J Obstet Gynecol.* 1998; 178(3):551-6; **Exhibit 1**; "Holden"), Ellis *et al.* (*Acta Obstr. Gynecol Scand* 2001,80,602-608; **Exhibit 2**; "Ellis") and Boger (WO 2002/114873, 2/21/2002; **Exhibit 3**; "Boger").

a) The References

Appellants recognize that the rejection encompasses a combination of the aforementioned references but nevertheless describe their teachings herein to reflect if or how their separate disclosures could be combined by one of skill in the art. Applicants address the combination of the references in Section VII.B.1.b.

Claim 1 concerns a method of determining that a pregnant woman is at risk of developing pre-eclampsia or that her fetus is at risk of developing intrauterine growth restriction (IUGR), by measuring asymmetric dimethylarginine (ADMA) in a plasma sample taken from a pregnant woman at 23 to 25 weeks gestation; the woman is at risk of developing pre-eclampsia or her fetus is at risk of developing IUGR if the level of ADMA in the sample is greater than 1.5 μmol/L. Claims 6-8 are dependent claims of claim 1.

Holden discusses ADMA levels in second and third trimester *normotensive* women (0.52 ± 0.20 μmol/L for the second trimester). Holden refrained from investigating ADMA levels in second trimester women yet determined in the third trimester that women who had been previously diagnosed with pre-eclampsia had ADMA levels of 1.17 ± 0.42 μmol/L. The skilled artisan would be taught from Holden that a pre-eclamptic woman has ADMA levels of 1.17 ± 0.42 μmol/L or higher in the third trimester. Holden does not teach anything about the levels of ADMA in women who are at an earlier stage of pregnancy and/or who are at risk of developing pre-eclampsia.

Concerning Holden, the Examiner states in the Final Office Action mailed December 9, 2010 ("the Action"; **Exhibit 4**), that Holden determined the level of ADMA to be around 0.52 to 1.17 during second trimester (first line, page 3 of the Action). This is not completely accurate, as all Holden demonstrates is that normal second trimester women were 0.52 μmol/L and pre-eclamptic third trimester women were 1.17 μmol/L (Appellants remove standard deviation for brevity). The Examiner also inaccurately characterizes Holden as stating that "...pregnant woman have [*sic*] pre-eclampsia if ADMA in the plasma sample is 1.25 gmol/L [*sic*]..." (the units are μmol/L); it is inaccurate because Holden states that the plasma ADMA concentration for third trimester pre-eclamptic women was 1.17 μmol/L (p. 554, left col.), not 1.25 μmol/L. In addition, the Examiner states on page 3 of the Action (line 5) that any ADMA level greater than 0.75 μmol/L would have pre-eclampsia and notes in the Interview Summary having a notification date of July 14, 2011 (**Exhibit 6**) that such a calculation derives from the teaching of Holden and is explained on page 9, line 4 of the Office Action mailed on September 22, 2009 (**Exhibit 7**). It is not clear to the Appellants how this is determined by the Examiner. However, such an extrapolation at least in Holden is irrelevant, because Holden teaches that pre-eclamptic women have ADMA levels of 1.17 ± 0.42 μmol/L or higher in the third trimester and is silent about any possible levels in the second

trimester for ascertaining risk for pre-eclampsia, particularly during the very specific weeks of 23-25 weeks gestation.

The Examiner notes that Holden fails to teach determination of ADMA level at 23-25 weeks of gestation and looks to Ellis for the deficiency. Ellis reviewed levels of ADMA and symmetric dimethylarginine (SDMA) in mild pre-eclamptic patients compared to severe preeclamptic patients and determined that "Symmetric, ***but not asymmetric***, dimethylarginine ***correlated to the severity*** of the condition." (emphasis added; Abstract). In other words, Ellis did not measure ADMA and SDMA predictively, only comparatively between normal and pre-eclamptic patients. Given this and the fact that Ellis teaches that "SDMA increased more than ADMA..." (page 607, right col.), the skilled artisan would be motivated on the basis of Ellis to employ SDMA levels (over ADMA levels) to more easily identify the presence of pre-eclampsia. The skilled artisan would not be motivated based on Ellis' teachings to determine ADMA levels and use them to predict whether or not a woman is at risk of developing pre-eclampsia, so Ellis would not be considered alone or in combination with any other reference to achieve the claimed invention.

In any event, Ellis discloses ADMA levels of 0.51-0.82 μmol/L (***no less, no more***) in pre-eclamptic women between 24-32 weeks of pregnancy (see Fig. 1). It is noteworthy that the Examiner makes the same statement on page 3, line 22, of the Action about Ellis that is made regarding Holden, namely that they allegedly teach ADMA levels greater than 0.75 μmol/L associated with pre-eclampsia, yet this is also inaccurate. The Examiner considers Ellis to differ from the claimed invention by "not measuring the ADMA level in non pre screened pregnant women" and refers to Boger, but Boger makes no mention of measuring any level of plasma ADMA in pregnant women during any stage of pregnancy, let alone suggest a level of 1.5 μmol/L or greater at a stage of pregnancy from 23 to 25 weeks.

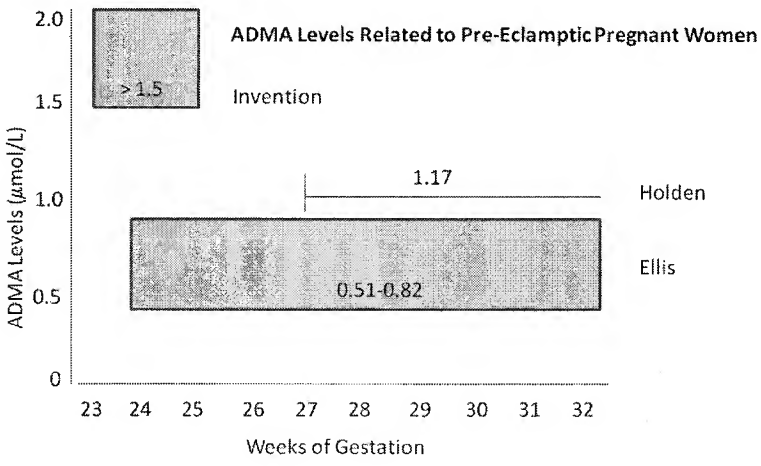
b) The Combination of References

Appellants assert that the claimed invention is patentable over the combination of references, because the combination does not teach, suggest, or provide motivation to modify the references to achieve the claimed invention. The Examiner contends on page 3 of the Action that it would have been obvious to combine Holden, Ellis, and Boger for measuring ADMA levels in detecting risk for pre-eclampsia for a woman at 23-25 weeks gestation. In particular, on page 4 of the Action the Examiner considered that one would have had reasonable expectation of success for the combination, because both Ellis and Holden taught methods of measuring ADMA plasma levels to determine a risk for pre-eclampsia and, furthermore, that the combination of Ellis, Holden, and Boger would have resulted in determining risk if the ADMA level is greater than normal, as allegedly suggested by Ellis.

To establish a *prima facie* case of obviousness, the prior art references when combined must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). The Holden, Ellis, and Boger references at the very least fail to suggest or teach to make the claimed combination. Obviousness requires a suggestion of all the elements in a claim (*CFMT, Inc. v. Yieldup Int'l Corp.*, 349 F.3d 1333, 1342 [68 USPQ2d 1940] (Fed. Cir. 2003)) and "a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1741 [82 USPQ2d 1385] (2007). As discussed below, the skilled artisan has no reason to combine the elements based on the combination, because they are incompatible.

Appellants provide the illustration below to highlight how differences between the references in the combination and differences with the claimed invention render the claimed

invention patentable under 35 USC §103(a), at least because the combination would require modification of one or more of the references to be unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification. *In re Gordon*, 733 F.2d 900, 221 USPQ 1125 (Fed. Cir. 1984).



As illustrated, the combination of Holden and Ellis are mutually exclusive with each other and also with the claimed invention, because they teach measurement of ***incompatible non-overlapping*** ADMA levels (1.17 µmol/L *vs.* 0.51 to 0.82 µmol/L *vs.* greater than 1.5 µmol/L). The skilled artisan would not obtain "greater than 1.5 µmol/L" from teachings of 1.17 µmol/L **or** 0.51 to 0.82 µmol/L. These are irreconcilable values, and the skilled artisan would not know which value to modify for the combination and whether the level should be raised from 0.82 µmol/L or reduced from 1.17 µmol/L, for example. One would have no reasonable expectation of success if the

combined references are incompatible and give the skilled artisan no guidance which of the references would be prevailing.

Furthermore, the skilled artisan would not include Holden's teachings in relation to the claimed invention, because it has ***mutually exclusive*** gestational windows for the third trimester with the claimed invention's requirement for 23 to 25 weeks. The skilled artisan would not be motivated to alter Holden for such a clear violation of its teaching. The skilled artisan from the combination of references including at least Holden would not know whether to monitor pre-eclampsia in the second trimester or third trimester.

The Examiner argues that Ellis' teaching ADMA level of 0.8 μmol/L would lead the skilled artisan to assume that a higher level of ADMA would put the woman at risk of developing pre-eclampsia (page 6 of the Action). However, Ellis must be considered in the conflicting combination with Holden and Boger, so would the combined teachings be at or around 1.17 μmol/L, within 0.51-0.82 μmol/L, or greater than 0.8 μmol/L, and would it be in the second or third trimester? In any event, Appellants assert that the representation is not necessarily correct, as Ellis' tested gestational period of 24-32 weeks is outside the claimed invention of 23 to 25 weeks. Furthermore, even if the skilled artisan would assume that levels higher than 0.8 μmol/L would suggest a risk of pre-eclampsia, there is no indication in the combination of references at which level the risk would be indicative. Would it be greater than 0.9 μmol/L, or more? Is there a threshold? The skilled artisan could not be aware of the limit of the level other than what is provided by the specification, so this is improper hindsight reasoning, and under *KSR* the skilled artisan must have a reason to modify the references in the combination to achieve a particular ADMA level.

2. Claims 1 and 11

Claims 1 and 11 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Holden, Ellis and Boger as applied to claims 1 and 6-8, and further in view of Albaiges *et al.* (*Obstet Gynecol* 2000;96:559-64; **Exhibit 5**; "Albaiges"). Claim 1 is referred to above, and claim 11 concerns the method further comprising carrying out Doppler waveform analysis of the uterine arteries and/or flow-mediated dilatation of the brachial artery in the woman. Albaiges concerns use of Doppler diagnosis of pre-eclampsia. Applicants note that claim 1 is included in the rejection with Albaiges, but claim 1 lacks any element concerning Doppler waveform analysis. Albaiges fails to add any teaching of pre-eclamptic ADMA levels to the combination of Holden, Ellis and Boger.

VIII. CONCLUSION

Appellants respectfully submit that the rejected claims are patentable over the cited art for the above-argued reasons. Appellants request that the Board of Patent Appeals and Interferences reverse the rejection of all claims.

Respectfully submitted,

/Melissa L. Sistrunk/

Melissa L. Sistrunk
Reg. No. 45,579
Attorney for Appellants

FULBRIGHT & JAWORSKI L.L.P.
1301 McKinney, Suite 5100
Houston, Texas 77010
(713) 651-3735
(713) 651-5246 (facsimile)

Date: September 8, 2011

IX. CLAIMS APPENDIX

1. A method of determining that a pregnant woman is at risk of developing pre-eclampsia or that her fetus is at risk of developing intrauterine growth restriction (IUGR), which method comprises:

(a) measuring asymmetric dimethylarginine (ADMA) in a plasma sample taken from a pregnant woman at a stage of pregnancy from 23 to 25 weeks gestation; and

(b) determining that the woman is at risk of developing pre-eclampsia or her fetus is at risk of developing IUGR if the level of ADMA in the plasma sample is greater than 1.5 μmol/L.

6. The method of claim 1, wherein determining that the woman is at risk of developing pre-eclampsia or determining that her fetus is at risk of developing IUGR comprises determining that the woman's ADMA level is at least 3 times the normal pregnancy level.

7. The method of claim 1, wherein determining that the woman is at risk of developing pre-eclampsia or determining that her fetus is at risk of developing IUGR comprises determining that the woman has an increase in the ADMA/symmetric dimethylarginine (ADMA/SDMA) ratio that is greater than the normal pregnancy ratio.

8. The method of claim 7, comprising determining that the ADMA/SDMA ratio is at least 5 times more than the normal pregnancy ratio.

11. The method of claim 1, further comprising carrying out Doppler waveform analysis of the uterine arteries and/or flow-mediated dilatation of the brachial artery in the woman.

X. EVIDENCE APPENDIX

Exhibit 1. Holden *et al.* (*Am J Obstet Gynecol.* 1998; 178(3):551-6, made of record by the Examiner in the Office Action mailed October 18, 2007

Exhibit 2. Ellis *et al.* (*Acta Obstr. Gynecol Scand* 2001,80,602-608, made of record by the Examiner in the Office Action mailed February 20, 2009

Exhibit 3. Boger, WO 2002/114873, made of record by the Examiner in the Office Action mailed February 20, 2009

Exhibit 4. Final Office Action mailed December 9, 2010

Exhibit 5. Albaiges *et al.* (*Obstet Gynecol* 2000;96:559-64, made of record by the Examiner in the Office Action mailed June 23, 2010

Exhibit 6. Interview Summary having a notification date of July 14, 2011

Exhibit 7. Office Action mailed on September 22, 2009

EXHIBIT 1

Plasma concentrations of asymmetric dimethylarginine, a natural inhibitor of nitric oxide synthase, in normal pregnancy and preeclampsia

Desmond P. Holden, MB, BS, Sara A. Fickling, PhD, Guy StJ. Whitley, PhD, and Stephen S. Nussey, DPhil
London, United Kingdom

OBJECTIVE: We investigated the change in the plasma concentration of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, in early-, mid-, and late-gestation normotensive pregnancies and in gestational age–matched preeclamptic pregnancies and compared the observed changes with changes in blood pressure.
STUDY DESIGN: Blood pressure and peripheral plasma asymmetric dimethylarginine concentrations were measured in 20 nonpregnant and 145 pregnant women (33 first-trimester, 50 second-trimester, and 44 third-trimester normotensive pregnancies and 18 third-trimester pregnancies complicated by preeclampsia). In 23 normotensive pregnancies serial plasma asymmetric dimethylarginine concentrations were measured. Statistical analysis was by analysis of variance and linear regression.
RESULTS: The blood pressures recorded throughout normal pregnancy were significantly lower than in nonpregnant subjects ($p < 0.0001$). The mean systolic, diastolic, and average blood pressures were significantly higher in the second-trimester groups than in the first-trimester groups, whereas in the third trimester average and diastolic blood pressures were significantly higher than in the second trimester. The mean (±SD) systolic and diastolic blood pressures in third-trimester preeclamptic patients was 157.7 ± 11.2 and 110.9 ± 8.5 mm Hg. The mean plasma asymmetric dimethylarginine concentration in nonpregnant women was 0.82 ± 0.31 μmol/L (significantly higher than in normotensive pregnancy, $p < 0.0001$). The plasma asymmetric dimethylarginine concentration was also significantly higher in second-trimester than in first-trimester normotensive groups (respectively, 0.52 ± 0.20 μmol/L and 0.40 ± 0.15 μmol/L, $p = 0.001$) and was higher in third-trimester normotensive pregnancy 0.56 ± 0.23 μmol/L than it was in the second trimester. The asymmetric dimethylarginine concentration in third-trimester preeclamptic patients was 1.17 ± 0.42 μmol/L ($p < 0.0001$ vs normotensive third-trimester subjects).
CONCLUSIONS: It is well recognized that blood pressure falls in early normal pregnancy and rises again toward term. These studies show that the early fall in blood pressure is accompanied by a significant fall in the plasma asymmetric dimethylarginine concentration. Later in pregnancy circulating concentrations increase and, when pregnancy is complicated by preeclampsia, concentrations are higher than in the nonpregnant state. Our data support a role for both asymmetric dimethylarginine and nitric oxide in the changes in blood pressure seen in both normal and preeclamptic pregnancy. (Am J Obstet Gynecol 1998;178:551-6.)

Key words: Nitric oxide, dimethylarginine, pregnancy (human), blood pressure, preeclampsia

In spite of advances in perinatal care preeclampsia remains a major cause of maternal and neonatal morbidity and mortality. Although the etiology of preeclampsia remains obscure, there is increasing evidence that its pathophysiologic basis is the activation of, and damage to, the maternal vascular endothelium. Various authors have shown that plasma or serum from pregnancies complicated by preeclampsia contains increased concentrations of markers for vascular endothelial damage such as fibronectin[1] and intercellular adhesion molecules.[2, 3]

Preeclampsia is characterized by vasospasm and a loss of the normal pregnancy-associated insensitivity to pressors.[4] Recently much work has focused on the role of nitric oxide, a free radical gas with many intracellular and intercellular signaling functions, in the maintenance of normotension in pregnancy. Nitric oxide is synthesized from L-arginine by the enzyme nitric oxide synthase, which exists as three isoforms, two of which are constitutively expressed and respond to agonist stimulation by

From the Department of Cellular and Molecular Sciences, St George's Hospital Medical School.
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Reprint requests: D. Holden, MB, BS, Department of Cellular and Molecular Sciences, St. George's Hospital Medical School, London, United Kingdom SW17 0RE.

0002-9378/98 $5.00 + 0 6/1/84983

Table I. Patient details and blood pressure measurements

	Nonpregnant (n = 20)	*First trimester (n = 33)*	*Second trimester (n = 50)*	*Third trimester (n = 44)*	*Preeclampsia (n = 18)*
Age (yr)	27.1 (5.4)	27.8 (5.1)	29.0 (5.1)	27.9 (5.3)	29.4 (6.1)
Parity	0	0	0	0	0
Gestational age (wk)	NA	10.1 (1.4)	20.0 (2.9)	32.6 (3.8)	32.7 (3.2)
Systolic BP (mm Hg)	120.7 (8.5)	100.9 (9.7)*†	111.1 (12.1)	115.0 (11.7)	157.7 (11.2‡
Diastolic BP (mm Hg)	78.9 (6.8)	62.4 (8.2)*§	67.7 (8.4)	72.2 (8.3)§	110.9 (8.5)‡

Values given are mean ± SD. *NA*, Not applicable; *BP*, blood pressure.
*$p < 0.0001$, compared with nonpregnancy values.
†$p = 0.0002$, compared with second-trimester values.
‡$p < 0.0001$, compared with values in all other groups.
§$p = 0.013$, compared with second-trimester values.

changes in activity, whereas the third isoform is induced in response to inflammatory cytokines. Nitric oxide synthase is competitively inhibited by guanidino-substituted arginine analogs, including N^GN^G-dimethylarginine (asymmetric dimethylarginine, ADMA) or N^G-methyl-L-arginine (L-NMMA).[5] Both ADMA and L-NMMA are found in human plasma and urine,[6] although ADMA is present at a more than tenfold higher concentration.

Rat and ovine pregnancy are associated with an increase in excretion of the breakdown products of nitric oxide and its second messenger cyclic guanosine monophosphate (cGMP).[7, 8] When nitro-L-arginine methyl ester, another competitive inhibitor of nitric oxide synthase, is administered to pregnant rats, a condition similar to human preeclampsia ensues with hypertension, proteinuria, platelet activation, and fetal intrauterine growth restriction. These changes are fully reversible after administration of excess L-arginine, suggesting that impaired nitric oxide production is a key factor.[9] Although the data from human pregnancy are conflicting owing to methodologic difficulties, there is evidence for increased nitrite and cGMP excretion in the urine in at least one pregnant population.[10] In a preliminary report we have shown that the concentration of ADMA in plasma taken from third-trimester normotensive pregnancies is lower than that in either nonpregnant controls or in pregnancies complicated by preeclampsia.[11] We have suggested that changes in the circulating concentration of this compound could be important in the pathophysiologic mechanisms of preeclampsia through a reduction in nitric oxide synthesis. We report here the changes in the plasma concentration of ADMA with increasing gestational age in normal pregnancy and the relationship between these concentrations and gestational changes in blood pressure. We have also measured the plasma ADMA concentrations in a larger group of pregnancies complicated by preeclampsia to assess further the differences between values in these patients and those in normotensive pregnant women and healthy, nonpregnant control subjects.

Methods

Subjects. The patients participating in this study were recruited from the antenatal clinics and the inpatient wards at St George's Hospital. All subjects gave informed consent and the study protocol had the approval of the local Ethics Committee. Preeclampsia was defined by the criteria of the U.S. National Institutes of Health Working Group on Hypertension in Pregnancy: all patients were primigravid, with proteinuria of ≥0.3 gm per 24 hours and with blood pressures consistently >140/90 mm Hg. No patient had preexisting hypertension or proteinuria and all became normotensive by the 6-week-postpartum check. At the time of blood sampling, no patient included in the preeclampsia group had received any antihypertensive medication. All blood pressure recordings were made with a standardized technique. The subject was placed in a semirecumbent position, and with use of an appropriate size cuff and mercury sphygmomanometer blood pressure was measured by auscultation over the right brachial artery. The diastolic measurement was that pressure coinciding with the fifth Korotkoff sound. Twenty-three normotensive pregnant women were studied in both the second and third trimester. All others were studied once at the stage of pregnancy indicated. No subject included in the study was in labor at the time of sampling. The outcomes of all pregnancies defined as normotensive were verified as normal. The control subjects were healthy, nulliparous, nonpregnant women volunteers from the department who did not smoke and were not taking oral contraceptives. Blood pressure measurements were taken at the time of blood sampling for the nonpregnant volunteers and close to the time of sampling (which was at routine antenatal clinic visits) for the normotensive pregnancies. Most preeclamptic patients were recruited to the study from the antenatal ward, at which time their blood pressure (in common with the nonpregnant subjects and most of the normotensive patients) was measured by one of the authors (D.P.H.). Where preeclamptic patients were recruited from the labor ward or from the obstetric high-dependency unit.

the blood pressure measured by an experienced midwife using an identical technique and recorded on observation charts closest to the time of blood sampling was used. Where two blood pressure recordings were noted for an antenatal visit both were included. Mean arterial blood pressure (MAP) was defined as the diastolic value plus one third the pulse pressure.

Sample collection. A blood sample (4 ml) for the measurement of plasma concentrations of ADMA was taken into ethylenediaminetetracetic acid (EDTA) (0.054 ml of 0.34 mol/L EDTA) at recruitment. After centrifugation the plasma was stored at −20° C until assayed.

Analysis of samples. All chemicals were purchased from Sigma Chemical Company (Poole, Dorset, United Kingdom) and were of high-pressure liquid chromatographic or analytic grade. Samples were extracted as previously described.[5] Aliquots (1 ml) of plasma were loaded onto preconditioned, 2 ml Bond Elut benzene sulfonic acid columns (Anachem, United Kingdom.). The dimethylarginines were eluted with 50% ammonia in methanol and evaporated to dryness at 130° C under nitrogen. The plasma eluate was redissolved in 1 ml of distilled water and added to a similarly preconditioned Bond Elut carboxylic acid column. The dimethylarginines were eluted with 10% ammonia in methanol and again evaporated to dryness. The samples were reconstituted in 100 µl of distilled water, centrifuged at 12,000*g* for 5 minutes, and the supernatants were removed for analysis.

Identification of dimethylarginines was by high-performance liquid chromatography. Separation of ADMA and its symmetric isomer was achieved by injecting 20 µl of the extracted sample onto an ODS C_{18} analytic high-performance liquid chromatography column (Spherisorb, Phasesep) with use of an ion pair-based mobile phase. Absorbance of the eluate was determined at 200 nm. Concentrations of ADMA in the samples were determined by computerized integration of peak area and comparison with known standard solutions. These concentrations were corrected for extraction efficiency, estimated by the addition of 10 µg of L-NMMA added to each sample before the extraction procedure. Interassay and intraassay variation was <5%. Statistical analysis of the results was performed with use of analysis of variance with the Student–Newman-Keuls post hoc test and linear regression analysis. Statistical significance was assumed at the 0.05 level.

Results

Patient details are shown in Table I. Throughout normotensive pregnancy systolic, diastolic, and average blood pressures were significantly lower than the values obtained in the nonpregnant controls ($p < 0.0001$, Fig. 1, *A*, and Table I). Within the normal pregnancy group average and diastolic blood pressures were significantly

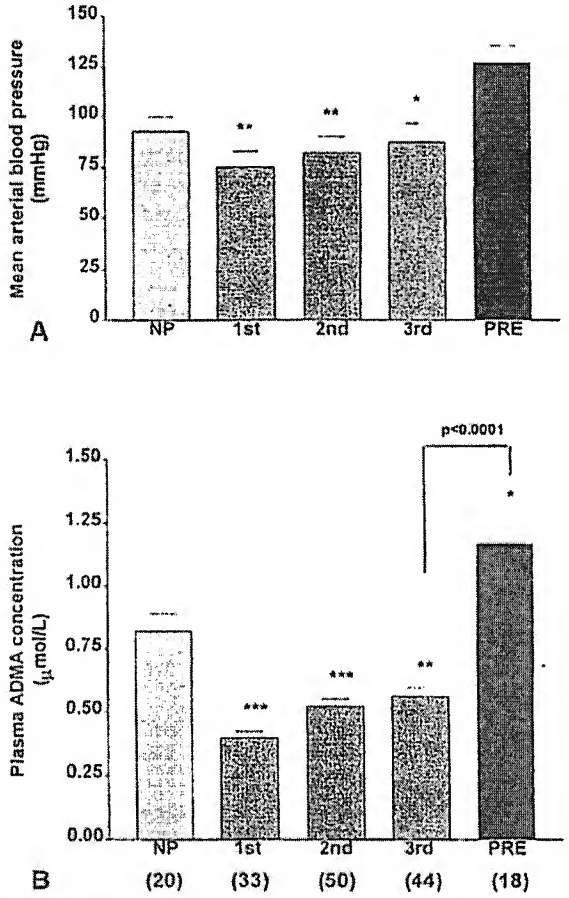

Fig. 1. A, MAP (mean ± SD) in nonpregnant volunteers (*NP*, $n = 20$); first-trimester ($n = 33$), second-trimester ($n = 50$), and third-trimester ($n = 44$) normotensive pregnant women; and third-trimester pregnancies complicated by preeclampsia (*PRE*, $n = 18$). In comparison with nonpregnant subjects, *two asterisks*, $p < 0.0001$; *asterisk*, $p = 0.039$. B, Mean (±SD) plasma ADMA concentration in same study groups. In comparison with nonpregnant group, *three asterisks*, $p < 0.0001$; *two asterisks*, $p = 0.0006$; *asterisk*, $p = 0.005$.

greater in the second trimester than in the first trimester ($p = 0.0001$ and $p = 0.013$, respectively) and in the third trimester than in the second trimester ($p = 0.005$ and $p = 0.013$, respectively). Systolic blood pressure was also significantly higher in the second trimester than in the first ($p = 0.0002$) and, although higher in the third trimester than in the second trimester, this did not reach statistical significance.

The plasma ADMA concentration (mean ± SD) of 33 first-trimester pregnancies was 0.40 ± 0.15 µmol/L. This was significantly lower than in both the nonpregnant control group (0.82 ± 0.31 µmol/L ($p < 0.0001$) and the second-trimester group of normotensive pregnancies (0.52 ± 0.20 µmol/L, $p = 0.003$). The mean plasma ADMA concentration was also higher in the third-

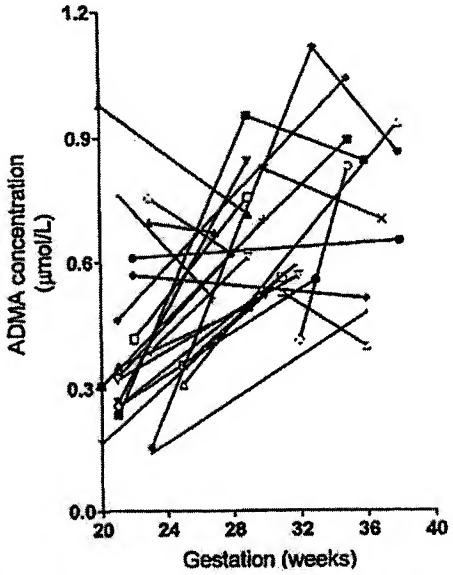

Fig. 2. ADMA concentration in serial plasma samples from normotensive pregnancies ($n = 23$). *Data points* are joined to show changes in individual study subjects.

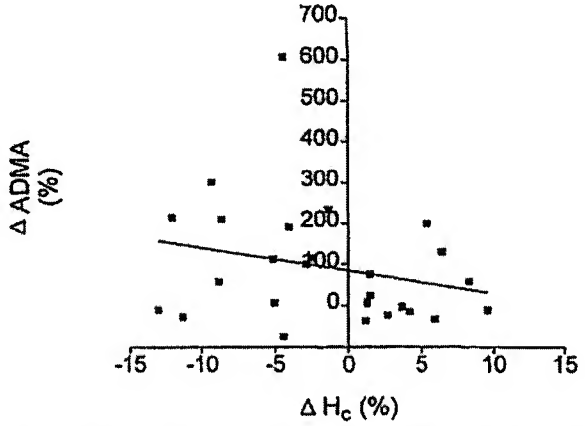

Fig. 3. Linear regression analysis of percentage change in hematocrit versus change in plasma ADMA concentration for subjects included in Fig. 2. Correlation coefficient, $r^2 = 0.05$; $p = 0.25$; $n = 26$.

trimester group than in the second-trimester group (0.56 ± 0.22 μmol/L) although this did not reach statistical significance at a 5% level. The mean plasma ADMA concentration for the 18 preeclamptic subjects was 1.17 ± 0.42 μmol/L, significantly greater than both the normotensive gestational age–matched and the nonpregnant control groups.

In the prospective study of 23 healthy normotensive pregnancies (Fig. 2) mean plasma ADMA levels rose from 0.43 ± 0.24 μmol/L in the second trimester to 0.68 ± 0.18 μmol/L in the third trimester ($p = 0.0001$). Linear regression analysis indicated that these changes in the plasma ADMA concentration within individual subjects were not correlated with changes in hematocrit ($r^2 = 0.05$, $p = 0.25$, Fig. 3).

The correlation between individual plasma ADMA concentrations and the systolic and diastolic and MAPs (Fig. 4) in our nonpregnant and normotensive pregnant populations was highly significant ($n = 147$, $p < 0.0001$). The coefficient of correlation, r^2, for the three relationships was, respectively, 0.25, 0.27 and 0.26. If the 18 preeclamptic subjects were included in the analysis, the correlation remained highly significant ($n = 165$, $p < 0.0001$), whereas the coefficient of correlation, r^2, for each blood pressure parameter with plasma ADMA concentration increased to 0.41.

Comment

Cardiac output increases by approximately 1.5 L/min in the first trimester of pregnancy, whereas the diastolic blood pressure falls by an average of 15 mm Hg to a nadir at 16 to 20 weeks' gestation, after which it rises toward prepregnancy values by term.[12] This fall in MAP is accomplished through a reduction in peripheral vascular resistance and a relative insensitivity to the elevated circulating concentrations of pressor agents such as angiotensin II.[13] This insensitivity may in part be related to increased circulating concentrations of progesterone, acting perhaps through the stimulation of vasodilator prostaglandin production.[14] Another candidate vasodilator is nitric oxide.[15] There is good evidence in animal pregnancy that the nitric oxide synthase activity of vascular tissues is increased.[16] In human pregnancy lack of control for dietary variation makes similar observations more difficult; however, studies have suggested a probable increase in the nitric oxide–cGMP pathway.[10] Thus the regulation of naturally occurring inhibitors of nitric oxide synthase activity could play an important role not just in the control of peripheral vascular resistance (and therefore blood pressure) in normal pregnancy but also in the pathologic alterations in blood pressure seen in preeclamptic toxemia.

ADMA is a good candidate as a physiologic regulator of nitric oxide synthase. Of the two naturally occurring methylarginines identified in biologic fluids that inhibit nitric oxide synthase activity, ADMA is present in at least a tenfold greater concentration. ADMA is released from vascular endothelial cells cultured in vitro.[17] In addition to its inhibitory effects on nitric oxide synthase, we have shown that it competes with L-arginine transport across endothelial cell membranes[18] and thus may alter nitric oxide production by reducing arginine availability. The enzyme methyltransferase I may be involved in ADMA synthesis by methylation of L-arginine in cellular protein.[19] A specific catabolic enzyme that metabolizes ADMA to citrulline (which has no ability to inhibit nitric

oxide synthase) has also been identified in human tissues. It is noteworthy that this enzyme, dimethylarginine diaminohydrolase, has been found to colocalize with sites of nitric oxide synthase expression, including vascular endothelial cells and placenta.[20]

Our results indicate that the reduction in plasma concentration of ADMA seen in normotensive pregnancy is accomplished by the end of the first trimester. Circulating plasma concentrations reflect the balance between the rates of synthesis and release of ADMA from cells, its reuptake into cells, and its metabolic clearance. In the absence of data on changes in these rates, it is difficult to be certain of the mechanisms behind the changes in plasma concentrations reported. The observation that the increase in ADMA concentration between the second and third trimesters in prospective studies did not correlate with changes in hematocrit suggests that the increase is not the result of alterations in circulating blood volume. Notwithstanding these caveats and the use of, for the most part, single measures of blood pressure and ADMA it is noteworthy that there is a statistically significant correlation between the plasma ADMA concentration and blood pressure at all stages of normal pregnancy.

We have shown that the circulating concentration of ADMA is high in third-trimester pregnancies complicated by preeclampsia. Given that endothelial cells may both release and take up ADMA, it is difficult to be certain what the differences in circulating ADMA concentration reflect at the actual sites of its actions. Studies with exogenous methylarginines indicate that the concentration differences measured may well be pathologically relevant. For example, intravenous infusion of L-NMMA (which is equipotent to ADMA) in human subjects with septic shock achieves a rise in average blood pressure of 20% at a plasma concentration of 3 μmol/L L-NMMA.[21] In addition, we have shown that 2 μmol/L ADMA significantly inhibits nitric oxide production in cell culture,[17] whereas Rutherford et al.[22] have shown that purified constitutive nitric oxide synthase binds ADMA with high affinity (inhibition constant for inhibition of N^G-nitro-L-arginine binding 649 nmol/L), indicating the potential relevance of submicromolar changes in local ADMA concentration.

The only other data of which we are aware on plasma ADMA concentrations in pregnancy have been obtained in the third trimester of Andean Ecuadorean patients with a high prevalence of preeclampsia.[10] With use of the same assay technique the values in nonpregnant women were identical to those in the current study; there was, however, no reduction in normal pregnancy and the values for the preeclamptic patients were no different. It is possible that the pathophysiologic mechanisms of preeclampsia are different in South London patients at sea level. This is supported by the fact that the plasma

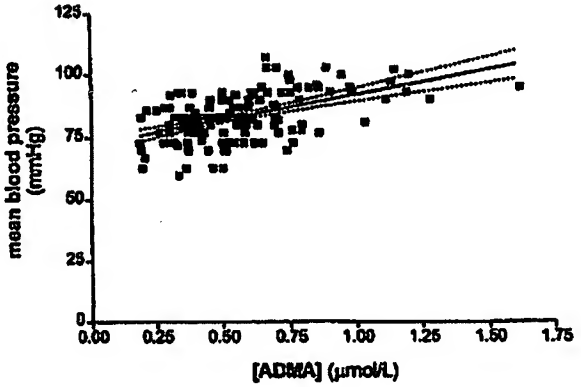

Fig. 4. Linear regression analysis (±95% confidence intervals) of plasma ADMA concentration versus MAP for nonpregnant and normotensive pregnant subjects. Correlation coefficient, $r^2 = 0.26$; $p < 0.0001$; $n = 147$.

atrial natriuretic factor concentrations in the Andean preeclamptic patients were no different from normotensive pregnancy values or, indeed, those of nonpregnant women, results that are at odds with other data.[23]

The gestational changes in plasma ADMA concentration that we have demonstrated both in normal and preeclamptic pregnancies may reflect an important method of regulating nitric oxide production. Further studies are required to elucidate the mechanisms underlying the changes in ADMA concentration during pregnancy. Data from prospectively collected samples are necessary to determine whether the changes in ADMA concentrations predate the clinical changes of preeclampsia and therefore support an etiologic role or, indeed, a clinically useful predictive role. From an etiologic point of view, samples taken early in the first trimester, when secondary trophoblast invasion is occurring, would be of particular interest.

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EXHIBIT 2

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ORIGINAL ARTICLE

Levels of dimethylarginines and cytokines in mild and severe preeclampsia

JOY ELLIS, ULLA-BRITT WENNERHOLM, ANDERS BENGTSSON, HÅKAN LILJA, ANDERS PETTERSSON, BO SULTAN, MARGARETA WENNERGREN AND HENRIK HAGBERG

From the Perinatal Center, Departments of Obstetrics and Gynecology, Anesthesiology and Clinical Pharmacology, Sahlgrenska University Hospital, Göteborg, Sweden

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Background. The objectives were 1. to evaluate if the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine was altered in mild and severe forms of preeclampsia, and 2. to assess the relationship between dimethylarginines and the cytokine response in preeclampsia.
Methods. Asymmetric and symmetric dimethylarginine were measured with high performance liquid chromatography in women with mild (n=13) and severe (n=32) preeclampsia and in normotensive pregnant controls (n=20). Interleukin-4, -6, -8, -10 and tumor necrosis factor-α were analyzed by immunoassays in women with mild (n=8) and severe (n=17) preeclampsia and in normotensive pregnant controls (n=14). The Mann Whitney U-test and Spearman Rank test were used for statistical analysis.
Results. The plasma levels of dimethylarginine were increased in preeclamptic subjects. The elevation of symmetric dimethylarginine was more pronounced than that of asymmetric dimethylarginine. The control levels of interleukin-6, -8 and -10 were significantly higher at term than at gestational week 32–36. Interleukin-6 and -8 were significantly elevated in subjects with severe, but not mild, preeclampsia, whereas TNF-α and IL-10 were not significantly altered. Symmetric dimethylarginine levels correlated significantly with arterial blood pressure and serum levels of creatinine and uric acid. Dimethylarginine levels in plasma were, however, not related to the cytokine response.
Conclusions. Plasma concentrations of both asymmetric and symmetric dimethylarginine were significantly elevated both in mild and severe preeclampsia. Symmetric, but not asymmetric, dimethylarginine correlated to the severity of the condition. Plasma levels of interleukin-6 and -8 were also elevated in severe preeclampsia but no direct correlations were found between these cytokines and dimethylarginines.

Key words: asymmetric dimethylarginine; cytokines; dimethylarginines; preeclampsia; symmetric dimethylarginine

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Many theories have been presented regarding the etiology and pathogenesis of preeclampsia, a multi-faceted complication of pregnancy entailing considerable fetal and maternal morbidity and mortality (1). Healthy primiparas and women with multiple fetuses as well as women with underlying vascular illness, such as diabetes, essential hypertension and systemic lupus erythematosis, are at higher risk of contracting this complication (2). Some individuals appear predisposed to preec-

Abbreviations:
ADMA: asymmetric dimethylarginine; AST: aspartate aminotransferase; ALT: alanine aminotransferase; BP: blood press-

paired placentation; i.e. defective invasion of the uterus by the trophoblast (2–4). The vascular changes accompanying normal pregnancy, replacement of the spiral arteries' muscle and elastic tissue by trophoblast, resulting in progressive arterial dilation and increased blood flow, are less pronounced or absent. Impaired placentation may result in relative or absolute hypoxia, atherosis-like lesions in placental and systemic vessels and impaired balance in immunological systems involving complement and cytokines. In a recent review, the hypothesis was put forward that pregnancy itself might be described as an inflammatory condition and that preeclampsia might be viewed as the result of an excess of inflammatory stimuli, i.e. one extreme of a continuum rather than a separate pathological entity (2).

Under normal conditions, cytokine levels may vary in different compartments in the pregnant body as well as during different stages of pregnancy, delivery and puerperium. While some researchers have observed elevated levels of various cytokines (IL-2, IL-4, IL-6, IL-8, TNF-α, IL-12) and their respective receptors in amniotic fluid and/or maternal blood both before and during onset of the clinical manifestations of preeclampsia (3–9), others have failed to observe such elevations or indeed observed the reverse (5, 7, 10–12). Some studies also report different cytokine-responses in different body fluids (13). This raises the issue of whether altered concentrations of various cytokines are a cause/mediator or a consequence of a pathological condition such as preeclampsia.

Numerous studies (14–22) have focused on the role played by nitric oxide (NO) and its synthesis inhibitors in these pathological processes. The amino acid L-arginine is transformed into the amino acid citrulline and nitric oxide (NO) by the enzyme nitric oxide synthase (NOS). NOS is partially regulated by negative feedback by NO, but there are also other important inhibitors that are present endogenously in man like the competitive inhibitor asymmetric dimethyl arginine, ADMA (asymmetric N (G),N (G)-dimethyl arginine) (23, 24). ADMA is synthetized from symmetric dimethyl arginine, SDMA symmetric (N (G), N'(G)-dimethyl-L-arginine) which also competes for L-arginine transport, but apparently does not block the enzymatic activity of NOS (25, 26). SDMA levels are elevated in end-stage renal failure, in dialysis patients and in hypertensive children (27–30). It is unclear whether this substance is merely an inert marker for some conditions involving renal dysfunction or if it is biologically active. The ADMA/SDMA quotient was found to be higher in preeclamptic subjects than in controls (22) whereas in uremic patients the ADMA/SDMA ratio was lower than in control subjects (28, 29). NO's role as a vasodilator, and consequences of NOS inhibition, have come into focus when the pathogenesis of preeclampsia is discussed. However, different research groups have arrived at disparate conclusions. Some studies have shown a decrease in NO and its metabolites early or late in preeclamptic pregnancies, and in such a scenario, an increased ADMA level may be of pathophysiological significance (22). Furthermore, NO metabolism is linked to the cytokine network. Calcium-independent NOS can, for example, be induced by some cytokines, most noticeably TNF-α and IL-1β (31–33). Other cytokines have an inhibitory effect on NOS, and synergistic effects of different cytokine combinations have been suggested (34). Furthermore, there is data indicating that NO may regulate production of some cytokines (35).

The aim of this study was to investigate the following two hypotheses:

1) ADMA levels would be higher in preeclamptic than in normal pregnancies whereas SDMA levels would be unaffected.

and

2) ADMA levels would correlate directly with levels of pro-inflammatory cytokines (IL-6, IL-8, TNF-α) and inversely with anti-inflammatory cytokines (Il-4, IL-10).

Methods

Thirteen women with mild and 32 women with severe preeclampsia participated and 20 women with normal, non-preeclamptic pregnancies were recruited as controls. Mild preeclampsia was defined as blood pressure (BP) exceeding 140/90 but not exceeding 160/110, in the presence of significant albuminuria (>0.3 g/24 h), with onset after 20 full weeks of pregnancy. Severe preeclampsia was defined as BP exceeding 160/110 and/ or albuminuria exceeding 5 g/24 h and/or laboratory results indicating affection of liver, kidney or coagulation system with onset after 20 completed weeks of pregnancy.

Women with multiple gestations, diabetes, hypertension or other underlying illness were excluded. Controls were tested at four-week intervals beginning at week 24 and ending at term. Preeclamptic participants were tested when the diagnosis was made, when there was any deterioration in their condition and in connection with delivery. Samples from the patients with severe preeclampsia were divided into two gestational age groups, 24–32 weeks (n=12) and 36–40 weeks (n=19). Onset of mild preeclampsia occurred at the latter gestational age, thus samples from this group of pa-

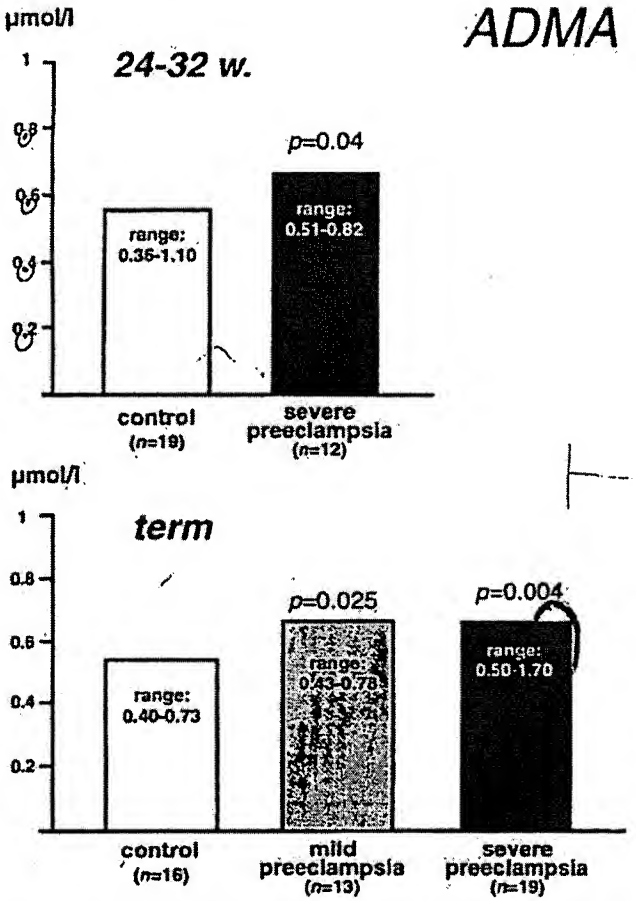

Fig. 1. The plasma concentration (µmol/l) of ADMA in mild and severe preeclampsia at 24–32 (upper panel) and at term (lower panel). Data are given as median (range) and comparisons *versus* control were performed by Mann-Whitney U-test.

tients were only taken at this stage in pregnancy. Patients gave informed consent after being provided with oral and written information and the study was approved by the Ethics Committee at Göteborg University (no: 532–99).

ADMA, SDMA, platelets, hemoglobin, creatinine, uric acid, AST, ALT, systolic and diastolic blood pressure and urine dipstick were tested at each occasion. ADMA and SDMA samples were divided into two gestational periods: weeks 24–32 and weeks 36–40 (term), respectively. No samples were taken from subjects with mild preeclampsia during the former gestational period since onset of mild preeclampsia essentially always occurs late in pregnancy.

Serum levels of cytokines were also tested in 17 subjects with severe preeclampsia and eight subjects with mild preeclampsia in connection with delivery, and in 14 controls, at four-week intervals fuged and stored at −80° C until analyzed. ADMA and SDMA were analyzed with high performance liquid chromatography, as described previously (36). Cytokine levels were analyzed in plasma by ELISA immunoassay (Quantikine™, R&D, United Kingdom). Detection limits were as follows: 0.7 pg/ml, <10 pg/ml, <2 pg/ml and 4.4 pg/ml for IL-6, IL-8, IL-10 and TNF-α, respectively.

Statistics: All values are given as median and range. Significance between groups was tested with the non-parametric Mann-Whitney U-test. Correlations were assessed with the Spearman Rank test.

Results

Median ADMA levels were higher both in subjects with severe and mild preeclampsia than in controls ($p=0.004$ and $p=0.025$, respectively) (Fig. 1), when measured at 36–40 weeks of gestation. The difference between ADMA levels in subjects with severe

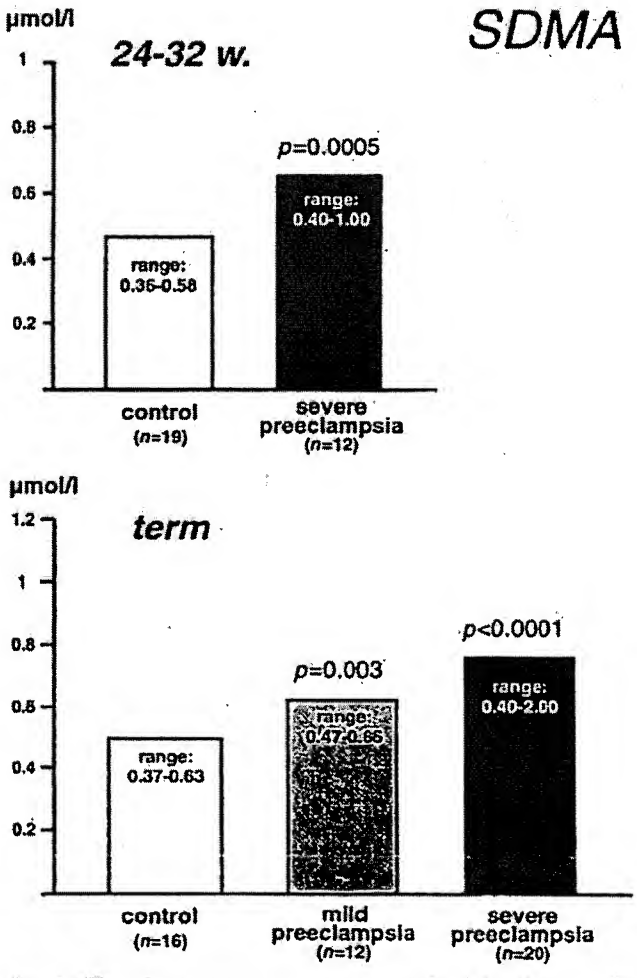

Fig. 2. The plasma concentration (µmol/l) of SDMA in mild

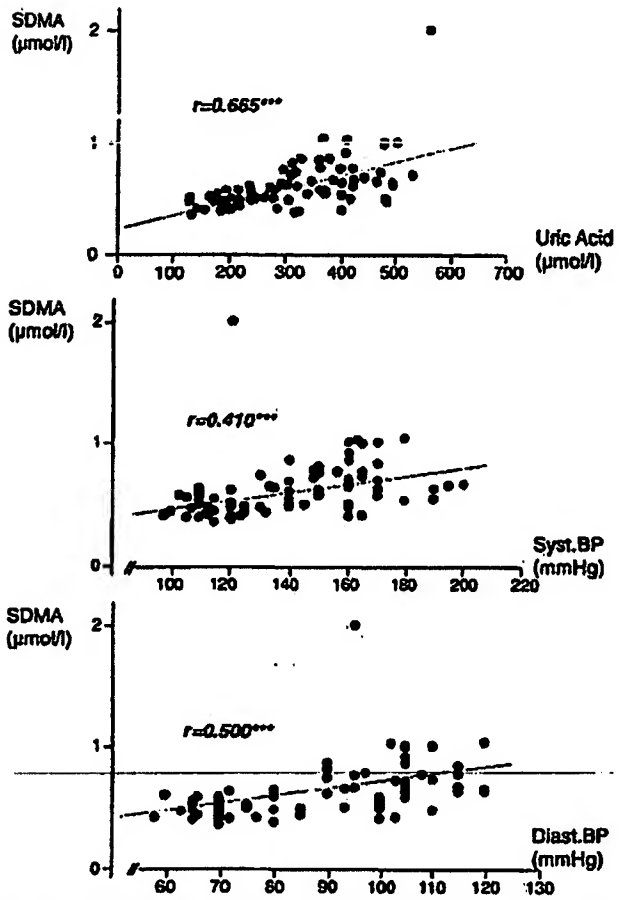

Fig. 3. Relationship between the plasma concentration (µmol/l) of SDMA and uric acid (µmol/l), systolic blood pressure (Syst.BP, mmHg) and diastolic blood pressure (Diast.BP, mmHg) for entire group, including both controls and preeclamptic subjects. The (Pearson) correlation coefficients (r) are given in the figure. Statistical analysis was performed with Spearman rank (each individual only contributing once); ***$p<0.001$.

preeclampsia and controls was somewhat less pronounced when measured at 24–32 weeks of gestation ($p=0.04$).

These differences were even more pronounced for SDMA (Fig. 2); levels were significantly higher in preeclamptic subjects than in controls in both severe ($p<0.0001$) and mild ($p=0.003$) preeclampsia during gestational weeks 36–40 and when subjects with severe preeclampsia were compared to controls during gestational weeks 24–32 ($p=0.0005$).

The ADMA/SDMA quotient, which might reflect the respective degrees of biological activity of these substances, was significantly lower in subjects with severe preeclampsia (1.03) than controls (1.28) at 24–32 weeks ($p=0.007$) and at 36–40 weeks (0.89 and 1.09, respectively; $p=0.0004$) of gestation. When the relationships between ADMA and studied clinical/laboratory parameters were analyzed, we found a statistically significant correlation ($p=0.02$) between ADMA and platelet levels in the preeclampsia (mild and severe) group.

When the entire study material (controls and subjects) was analyzed together (Fig. 3), we found that SDMA levels correlated significantly with serum uric acid ($r=0.665$), systolic BP ($r=0.410$), diastolic BP ($r=0.500$) and the creatinine concentration in serum ($r=0.694$).

Table 1 shows cytokine levels at increasing gestational ages in controls. The levels generally surged at term: the levels of IL-6 ($p=0.005$), IL-8 ($p=0.007$) and IL-10 ($p=0.05$) were lower at 32–36 weeks than at 40 weeks of gestation. IL-8 and IL-6 were significantly elevated in severe, but not in mild, preeclampsia, compared to controls (Fig. 4). The levels of IL-10 and TNF-α tended to be higher in severe preeclampsia (Fig. 5) but the differences were not statistically significant.

IL-4 was not detected in any control or preeclamptic subject. IL-6 correlated significantly ($p=0.01$) with systolic BP in the preeclampsia group (mild and severe together) and there was a correlation between uric acid levels and IL-10 in the preeclampsia group ($p=0.009$). When the inter-relationships between each cytokine and ADMA or SDMA were studied, we found no statistically significant correlations in the preeclampsia group.

Discussion

Thus, our first hypothesis was both confirmed and refuted; both ADMA and SDMA were signifi-

Table 1. Levels of interleukin (IL)-6, 8, 10 and tumor necrosis factor (TNF)-α in control subjects at 24, 28, 32, 36 and 40 weeks of gestation. Values are expressed as medians (range). The levels of IL-6, IL-8 and IL-10 were significantly higher at 40 weeks than at 32-36 weeks of gestation. Significance levels: * $p\leq0.05$;** $p\leq0.01$

	24 weeks (n=6–12)	28 weeks (n=6–11)	32 weeks (n=9–12)	36 weeks (n=4–8)	40 weeks (n=6–9)
IL-6 (pg/mL)	0.0 (0–9.0)	0.4 (0.2–1.4)	0.6 (0–3.9)	0.0 (0–1.2)	14.7 (0.5–69.5)**
IL-8 (pg/mL)	0.7 (0–103.0)	0 (0–158.7)	0.4 (0–8.2)	2.2 (0–13.4)	13.2 (0–50.3)**
IL-10 (pg/mL)	4.9 (0–12.9)	4.4 (2.0–22.9)	3.6 (0–9.0)	4.8 (0–13.6)	7.0 (2.4–30.0)*
TNF-α (pg/mL)	2.2 (0.5–4.7)	2.2 (1.2–4.7)	3.5 (1.8–5.6)	3.1 (2.7–3.8)	3.7 (0.9–4.7)

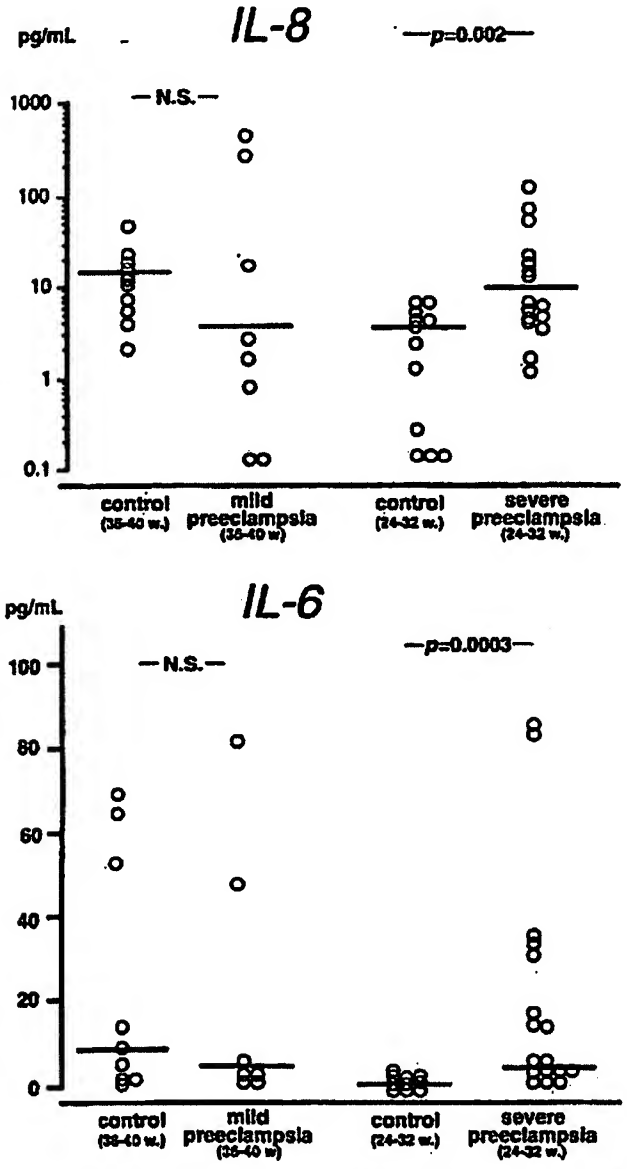

Fig. 4. The plasma concentration (pg/mL) of IL-8 and IL-6 are presented for each individual (circle) and median values (horizontal bars) for cases with mild and severe preeclampsia and for corresponding controls. The values of IL-8 are given on a log scale and values of IL-6 on a linear scale. Levels of significance were tested with Mann-Whitney U-test.

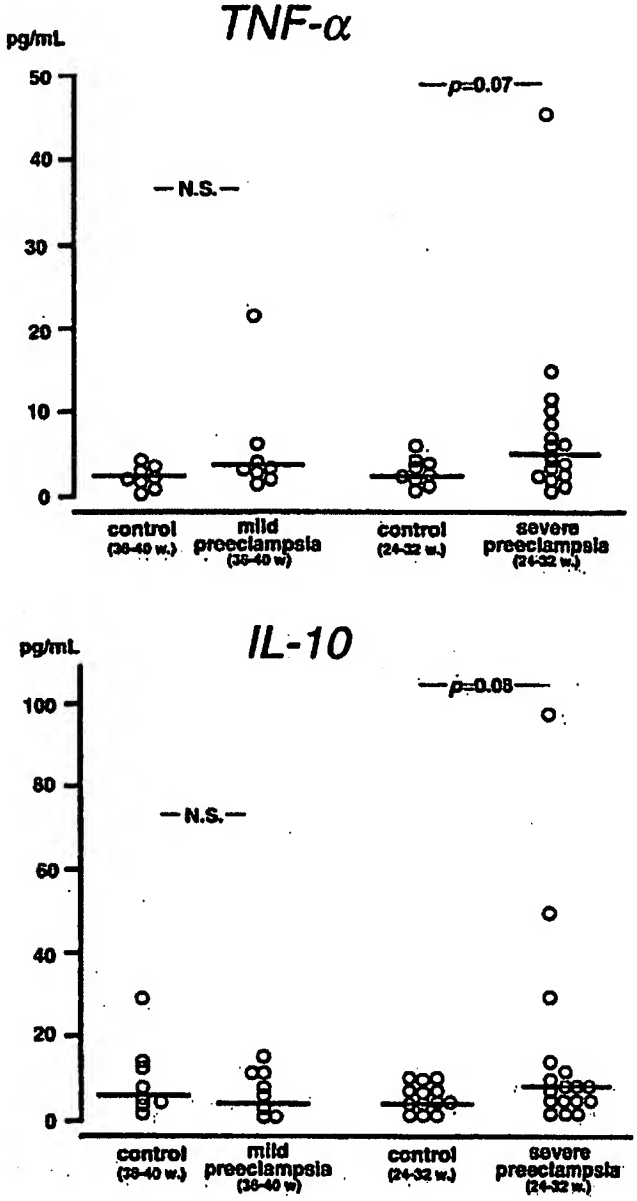

Fig. 5. The plasma concentration (pg/mL) of TNF-α and IL-10 are presented for each individual (circle) and median values (horizontal bars) for cases with mild and severe preeclampsia and for corresponding controls. Levels of significance were tested with Mann-Whitney U-test.

cantly higher in subjects with mild and severe preeclampsia compared to normotensive controls at appropriately matched gestational ages. There were no convincing correlations between ADMA and clinical parameters. However, the ADMA/SDMA quotient was significantly lower in subjects with severe preeclampsia than in controls, reflecting a marked elevation of SDMA. These re-

different types of renal strain and hypertension (27, 30), elevations which may or may not exceed ADMA increases in the corresponding clinical situations (28, 30). In light of the fact that normal pregnancy tends to cause a certain amount of renal strain as it progresses, a phenomenon which is even more pronounced in preeclamptic pregnancy, our results are, perhaps, not too surprising. In-

This evoked our curiosity as to whether SDMA might equal or be superior to uric acid as a marker of the severity of preeclampsia, but this proved not to be the case. However, it might be interesting to study ADMA and SDMA levels early in pregnancy in order to ascertain if levels rise early enough to predict preeclampsia.

The cytokines IL-6 and IL-8 were significantly elevated in subjects with severe, but not mild, preeclampsia compared to controls. This is in accordance with the work of some, but not all previous investigators. Greer et al. (7) found significantly elevated IL-6 levels but unaltered TNF-α and IL-8 levels in the plasma of preeclamptic subjects. Conrad et al. (9) found elevated levels of TNF-α and IL-6 but not IL-8 in the plasma of preeclamptic women, while Vince et al. (3) found significantly elevated plasma IL-6, TNF-α and soluble TNF receptor levels in the preeclamptic group. Kupferminc et al. found significant TNF-α elevations in plasma of preeclamptic subjects in one study (6), and the same group found elevated plasma levels of soluble TNF receptors and IL-6, the latter when measured prior to labor, in another study of preeclamptic women (13). On the other hand, Meekins et al. (10) failed to find a significant difference in plasma TNF-α levels when comparing normotensive pregnant women to preeclamptic subjects and Opsjön et al. (37) found no differences in maternal IL-6 or TNF blood levels when comparing these groups of women.

There is a considerable variation in normal cytokine levels in pregnant women reported by different authors and the overlap between normal and pathological states are noteworthy (1). These discrepancies may reflect different analysis methods (e.g. bioassay *vs* immunoassay), testing performed on laboring *vs* non-laboring women, gestational age or varying populations (1). As indicated in Table I, three of the four measured cytokines increased significantly at the end of pregnancy as delivery approached. Therefore, it is essential that the gestational age of the preeclampsia group is meticulously matched with that of the control group.

Our second hypothesis was not confirmed as none of the measured cytokines correlated to ADMA or SDMA. The cytokine network has, as yet, been incompletely characterized and inter-relationships among groups of cytokines and correlations to clinical disease are, to say the least, complex and elusive, since different research groups have arrived at diverging conclusions. Obviously, more research is needed to clarify these issues; based on the present study, it is not possible to rule out a link between the cytokine system and ADMA/SDMA.

Conclusion

In this study, we found increased levels of ADMA and SDMA in both mild and severe preeclampsia compared to normotensive controls at appropriately matched gestational ages. SDMA increased more than ADMA, resulting in lower ADMA/SDMA quotients in severe preeclampsia. Significantly higher levels of the cytokines IL-6 and Il-8 were found in the serum of women with severe preeclampsia compared to healthy pregnant controls. Control subjects demonstrated a statistically significant surge in levels of IL-6, IL-8, IL-10 but not TNF-α, at term, compared to gestational week 32–36. No convincing correlations between the NOS inhibitor ADMA and any cytokine were observed. A clinical marker predicting onset or degree of severity of preeclampsia would definitely be useful to the clinician but it is apparent that more light must be shed both on NO inhibitors and on the complex cytokine systems before either can serve in this function.

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Address for correspondence:

Henrik Hagberg, M.D., Ph.D.
Department of Obstetrics and Gynecology
Perinatal Center
Sahlgrenska University Hospital/Östra
416 85 Göteborg
Sweden

EXHIBIT 3

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Result Page

Description "method and means for the proof of a probability of the future occurrence or progressing of diseases, those with a disturbance of the NO of metabolism accompany."
The invention relates to methods for the proof of a probability of the future occurrence or progressing of diseases, which accompany with a disturbance of the NO metabolism. In addition the invention concerns means to the proof of endogenous methyl arginines in biological fluids.

The endogenous methyl arginines ADMA and SDMA are derivatives of the amino acid L arginine; L arginine is the preliminary stage to the formation of nitrogen monoxide (NO) in the human body. NO again is an important physiological mediator in the heart circulatory system and in other organ systems, which is involved at the regularization of blood pressure and container resistance, adhesion and aggregation of Thrombozyten, adhesion of leukocytes and monocytes and the proliferation of smooth Gefässmuskelzellen (Böger et al., Atherosclerosis 1996; 127 : 111). NO plays also an important physiological role with the erection. With heart circuit illnesses such as arteriosclerosis, hypercholesterolemia, hypertension, chronic heart failure, with metabolic illnesses such as diabetes mellitus, with Präeklampsie, with erektiler dysfunction and other diseases comes it to the attenuation of the biological effects of NO, whereby progressing of these diseases and the accompanying Gefässlasionen becomes accelerated. By administration of L arginine this happening counteracted can become.

In several clinical and experimental studies shown could become that it can come with the diseases mentioned to rising the concentration of the endogenous L-arginine-analogue ADMA into the plasma or serum. Increased concentrations of ADMA found during peripheral arterial catch illness (Böger et al., Circulation 1997; 95 : 2068-2074), with hypercholesterolemia (Böger et al., Circulation 1998; 98 : 1842-1847), with hypertension (Surdacki et al., J. Cardiovasc. Pharmacol. 1999 ; 33 : 652-658), with chronic kidney insufficiency (keel Kiel et al., J. To. Soc. Nephrol. 1999 ; 10 : 594600) and with chronic heart failure. A cause effect relationship between increased ADMA concentrations and these diseases could not become from the results of these studies however derived. ADMA is an inhibitor of the formation of NO from L arginine, which becomes by the enzyme NO synthase in Endotheizellen mediated. Thus it would be explainable that the increased concentrations of ADMA could contribute by inhibition of the NO formation to progressing the disease process. It leads also increased blood glucose concentrations, as they arise with the diabetes mellitus, to reduced effect of NO whereby the occurrence of complications heart of the circulatory system promoted becomes. With the Präeklampsie comes it by the disturbance of the NO metabolism to a constriction of arteries, which releases an hypertension with the nut and leads by less blood circulation of the placenta to the endangerment of the unborn child. Furthermore a lack of the effects of NO is a substantial cause of the erektilen dysfunction. On the other hand SDMA is likewise an endogenous occurring molecule, which however obviously exercises no inhibitive effect on the activity of the NO synthase.

▲ top The invention is the basis the observation that patients with hypercholesterolemia, peripheral arteriosclerosis, heart failure, chronic kidney insufficiency, diabetes mellitus and hypertension exhibit higher concentrations of ADMA in the plasma than healthy subjects. We could show now for the first time that ADMA is a factor prognostically relevant for future progressing of the diseases: Patients with higher ADMA concentrations have a significant higher probability to suffer a threatening blood circulation disturbance or decease to it than patients with lower ADMA concentrations (example 1). Patients with high ADMA concentrations have to decease also altogether a significant higher risk, to independent of the underlying cause of death (example 2).

Ferner findet sich bei Patienten mit höheren ADMA-Konzentrationen signifikant häufiger eine Verdickung der Arterienwand in der Halsschlagader (A. carotid) as with patients with low ADMA concentrations (example 3). Patients with chronic heart failure, which increased ADMA concentrations exhibit, have a smaller maximum oxygen admission bottom physical Belastung-ein reference on an adverse prognosis of the future disease process (example 4).

Since ADMA is thus mentioned a factor relevant for the disease process of the diseases, it is useful, the concentration with the individual patient by means of an universal available and rapid diagnostic test specific and they measure of the SDMA concentration to differentiate to be able.

At present available measuring methods to the quantitative determination of ADMA and SDMA in plasma, serum, urine and

other biological fluids (Gewebsextrakten, Zellkulturüberständen and. A.) are based all together on the chemical detection method of the high pressure Flüssigkeitschromatografie (HPLC).

Various modifications of HPLC methods become used at present for this.

These methods are however very zeit-und personnel-intense, expensively, and thus for the clinical routine diagnostics not suitable.

Object of the present invention is it to prove a probability of future progressing of diseases which accompany with a disturbance of the NO metabolism.

This object becomes according to invention dissolved by the fact that a content of endogenous methyl arginines in biological fluids becomes certain.

Despite the very circumferential determination of most diverse known cardiovascular factors of risk progressing the diseases mentioned is to be proven with a portion of patients despite absence of such known factors of risk. The invention exhibits the advantage that at least a part of these cases can become explained by being present increased concentrations of the endogenous methyl arginines and thus an improved prognosis can become discharged about the disease process.

The used detection methods for ADMA and SDMA are based on the principle of the HPLC, which makes them expensive for very expensive and, so that these methods remain being able to become not comprising in clinical everyday life used, but specialized research labs reserving. An other object of the invention is it to submit a method with whose assistance simple, inexpensive and universal clinical of the useful endogenous methyl arginines ADMA and SDMA quantitative in biological fluids certain to become to be able.

This object becomes according to invention dissolved by the fact that to the determination of the content by endogenous methyl arginines in a biological fluid on these with antibodies one influences.

Those, determination ADMA and of the SDMA concentrations by means of immunochemical methods (D. h. using monoclonal or polyclonal antibodies) has according to invention significant advantages: 1. The measurement ADMA and of the SDMA concentrations by means of use of anti-body-based methods does not have to become in high-specialized HPLC laboratories performed. Immunochemical detection methods how
Radioimmunoassay, enzyme immunoassay etc. routine are more available in most clinical chemical laboratories.

2. The measurement with immunochemical detection methods lasts fewer prolonged than the measurement by means of HPLC. For latter method is an expensive sample extraction from the respective matrix (plasma, serum, urine o. A.) required, so that the measurement can take place by HPLC. The rapid
Are present the measurement value is however for the use ADMA and/or.

SDMA proof in the clinical everyday life unavoidably necessary, thereby these
Parameter as disease marker wide acceptance to find can.

3. Immunochemical detection methods can come beside the clinical routine also into experimental applications to the use (z. B.

Immunohistochemie, Immunozytochemie, Immunoblotting).

▲ top 4. The corresponding present invention is the diagnostic proof of
ADMA and/or. SDMA suitable, over prospektiv over illness or
Death risk of an patient explanation to attain ("factor of risk").

The generation of monoclonal antibodies of made experimental animals with myeloma cells and selection, sensitized after allgemein known immunologic methods by fusion of immune-competent cells, the specific antibodies of producing cell clones by means of standard methods. The generation of polyclonal antibodies made by means of recovery of Immunserum of immunized experimental animals after general prior art methods. The production of monoclonal antibodies against ADMA and SDMA covers the cultivation of the anti cell clones, the recovery of the anti-body-contained of conditioned medium, as well as the filling and the selling of the antibody solutions. The use of the antibodies covers their use to diagnostic and scientific purposes, as anti body suspension or as component of a diagnostic kit, in the ranges of the clinical medicine, the experimental medicine, the veterinary medicine, the biology and other life sciences.

The appended examples describe the invention:
Example 1
225 patients with chronic kidney insufficiency became received into a clinical study. Starts of the study each patient a blood sample became removed, became certain in which the concentrations of ADMA and SDMA as well as of L arginine. The patients became over a middle observation duration of 26 months (1-35 months) after-observed. During this time 57 patients suffered a cardiovascular disease event (myocardial infarction, impact accumulation, peripheral blood circulation

disturbance or cardiac conditional death). Patients with such an cardiovascular disease event had had significant higher ADMA concentrations to starts of the study (median 3.2 umol/L) than patients without such event (median 2.2 umol/L). ADMA proved in the statistical analysis as an independent predictor of cardiovascular disease events.

Became the patients on the basis the ADMA Plasmakonzuentrationen in groups divided (ADMA, measured to starts of the study < 50. Percentiles, 51. - 70.

Percentiles, 71. - 90. Percentiles, and > 90.), Then a clear increase of the incidence of cardiovascular deaths with rising ADMA concentration (image 1) resulted percentiles.

Example 2
With 225 patients, with which in a blood sample the ADMA concentration certain removed to starts of the study was, on the average 26 months permanent Nachbeobachtungsphase arose altogether to 57 death trap due to most diverse causes in the course of one. Patients, which deceased in the course of the study, had significant higher ADMA concentrations to starts exhibited (median 3.5 umol/L) than patient, the survived (2.3 umol/L) ADMA proved than a predictor of the Überiebens independent of other factors of risk. Became the patients on the basis the ADMA measured to starts of the study plasma concentrations in groups divided (ADMA < 50. Percentiles, 51. - 70.

Percentiles, 71. - 90. Percentiles, and > 90.), Then a clear increase of the entire mortality with rising ADMA concentration (image 2) resulted percentiles.

Example 3
With 90 patients the ADMA concentration became certain in a plasma sample. Subsequent one became with this patients by means of highly soluble ultrasonic investigation the wall thickness of the carotid arteries (A. carotid) measure.

ADMA was an independent factor of influence for the Intima/Media thickness of the A. carotid (image 3), for the luminale cross-section area, and for the severeness of the arteriosklerotischen lesions in the A. carotid.

Example 4
With 45 patients with chronic heart failure a blood sample became removed. The measurement of the ADMA concentration resulted in an average value of 4,1 i 0.8 lumol/l, compared with 1,0,0.1 umol/l in the case of healthy control persons.

Patients with higher ADMA concentrations had a smaller NO-mediated Gefässdilatation (R =-0. 79) and a smaller maximum oxygen admission bottom physical load (R =-0. 81 ; Image 2a). The administration of L arginine guided to a significant improvement of the NO-mediated Gefässdilatation; the extent of this improvement by L arginine was the large, the per high ADMA concentration with the respective patient was (R = 0. 74 ; Image 2b).

▲ top

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(71) Anmelder und
(72) Erfinder: BÖGER, Rainer, H. [DE/DE]; Hartwicusstr. 2, 22087 Hamburg (DE).

(74) Anwalt: HELDT, Gert; Grosse Bleichen 12 I, 20354 Hamburg (DE).

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WO 02/14873 A2

(54) Title: METHODS AND AGENTS FOR DETECTING THE PROBABILITY OF THE FUTURE OCCURRENCE OR PROGRESSION OF DISEASES THAT ARE ASSOCIATED WITH A DISORDER OF THE NO METABOLISM

(54) Bezeichnung: VERFAHREN UND MITTEL ZUM NACHWEIS EINER WAHRSCHEINLICHKEIT DES ZUKÜNFTIGEN AUFTRETENS ODER FORTSCHREITENS VON ERKRANKUNGEN, DIE MIT EINER STÖRUNG DES NO-STOFFWECHSELS EINHERGEHEN

(57) Abstract: The invention relates to methods and agents for detecting the probability of the future occurrence or progression of diseases that are associated with a disorder of the NO metabolism.

(57) Zusammenfassung: Verfahren und Mittel zum Nachweis einer Wahrscheinlichkeit des zukünftigen Auftretens oder Fortschreitens von Erkrankungen, die mit einer Störung des NO-Stoffwechsels einhergehen.

Beschreibung

"Verfahren und Mittel zum Nachweis einer Wahrscheinlichkeit des zukünftigen Auftretens oder Fortschreitens von Erkrankungen, die mit einer Störung des NO-Stoffwechsels einhergehen."

Die Erfindung betrifft Verfahren zum Nachweis einer Wahrscheinlichkeit des zukünftigen Auftretens oder Fortschreitens von Erkrankungen, die mit einer Störung des NO-Stoffwechsels einhergehen. Darüberhinaus betrifft die Erfindung Mittel zum Nachweis von endogenen Methyl-Argininen in biologischen Flüssigkeiten.

Die endogenen Methyl-Arginine ADMA und SDMA sind Derivate der Aminosäure L-Arginin. L-Arginin ist die Vorstufe zur Bildung von Stickstoffmonoxid (NO) im menschlichen Körper. NO wiederum ist ein wichtiger physiologischer Mediator im Herz-Kreislaufsystem und in anderen Organsystemen, der an der Regulation von Blutdruck und Gefäßwiderstand, Adhäsion und Aggregation von Thrombozyten, Adhäsion von Leukozyten und Monozyten und der Proliferation glatter Gefäßmuskelzellen beteiligt ist (Böger et al., Atherosclerosis 1996; 127: 1-11). NO spielt auch eine wichtige physiologische Rolle bei der Erektion. Bei Herz-Kreislauferkrankungen wie Arteriosklerose, Hypercholesterinämie, Hypertonie, chronischer Herzinsuffizienz, bei Stoffwechselerkrankungen wie Diabetes mellitus, bei Präeklampsie, bei erektiler Dysfunktion und anderen Erkrankungen kommt es zur Abschwächung der biologischen Wirkungen von NO, wodurch das Fortschreiten dieser Erkrankungen und der begleitenden Gefäßläsionen beschleunigt wird. Durch Gabe von L-Arginin kann diesem Geschehen entgegengewirkt werden.

In mehreren klinischen und experimentellen Untersuchungen konnte gezeigt werden, daß es bei den genannten Erkrankungen zu einem Ansteigen der

Konzentration des endogenen L-Arginin-Analogons ADMA im Plasma oder Serum kommen kann. Erhöhte Konzentrationen von ADMA wurden gefunden bei peripherer arterieller Verschlußerkrankung (Böger et al., Circulation 1997; 95: 2068-2074), bei Hypercholesterinämie (Böger et al., Circulation 1998; 98: 1842-1847), bei Hypertonie (Surdacki et al., J. Cardiovasc. Pharmacol. 1999; 33: 652-658), bei chronischer Niereninsuffizienz (Kielstein et al., J. Am. Soc. Nephrol. 1999; 10: 594-600) und bei chronischer Herzinsuffizienz. Eine Ursachen-Wirkungsbeziehung zwischen erhöhten ADMA-Konzentrationen und diesen Erkrankungen konnte aus den Ergebnissen dieser Studien jedoch nicht abgeleitet werden. ADMA ist ein Inhibitor der Bildung von NO aus L-Arginin, die durch das Enzym NO-Synthase in Endothelzellen vermittelt wird. Somit wäre erklärlich, daß die erhöhten Konzentrationen von ADMA durch Hemmung der NO-Bildung zum Fortschreiten des Krankheitsprozesses beitragen könnten. Auch erhöhte Blut-Glucose-Konzentrationen, wie sie beim Diabetes mellitus auftreten, führen zu verminderter Wirkung von NO, wodurch das Auftreten von Komplikationen des Herz-Kreislaufsystems gefördert wird. Bei der Präeklampsie kommt es durch die Störung des NO-Stoffwechsels zu einer Verengung von Arterien, die einen Bluthochdruck bei der Mutter auslöst und durch Minderdurchblutung der Plazenta zur Gefährdung des ungeborenen Kindes führt. Ferner ist ein Mangel der Wirkungen von NO eine wesentliche Ursache der erektilen Dysfunktion. Dagegen ist SDMA zwar ebenfalls ein endogen vorkommendes Molekül, welches aber offenbar keine inhibitorische Wirkung auf die Aktivität der NO-Synthase ausübt.

Der Erfindung liegt die Beobachtung zugrunde, daß Patienten mit Hypercholesterinämie, peripherer Arteriosklerose, Herzinsuffizienz, chronischer Niereninsuffizienz, Diabetes mellitus und Hypertonie höhere Konzentrationen von ADMA im Plasma aufweisen als gesunde Probanden. Wir haben nunmehr erstmals zeigen können, daß ADMA ein für das zukünftige Fortschreiten der Erkrankungen prognostisch relevanter Faktor ist: Patienten mit höheren ADMA-Konzentrationen haben eine signifikant höhere Wahrscheinlichkeit, eine bedrohliche Durchblutungsstörung zu erleiden oder daran zu versterben als Patienten mit niedrigeren ADMA-Konzentrationen (Beispiel 1). Patienten mit hohen ADMA-Konzentrationen haben auch insgesamt ein signifikant höheres Risiko zu versterben, unabhängig von der zugrundeliegenden Todesursache (Beispiel 2). Ferner findet sich bei Patienten mit höheren ADMA-Konzentrationen signifikant häufiger eine Verdickung der Arterienwand in der Halsschlagader (A. carotis) als bei Patienten mit niedrigen ADMA-Konzentrationen (Beispiel 3). Patienten mit chronischer Herzinsuffizienz, die erhöhte ADMA-Konzentrationen aufweisen, haben eine

geringere maximale Sauerstoffaufnahme unter körperlicher Belastung – ein Hinweis auf eine ungünstige Prognose des zukünftigen Krankheitsverlaufs (Beispiel 4).

Da ADMA somit ein für den Krankheitsverlauf der genannten Erkrankungen relevanter Faktor ist, ist es nützlich, die Konzentration beim individuellen Patienten mittels eines universell verfügbaren und raschen diagnostischen Tests spezifisch messen und sie von der SDMA-Konzentration unterscheiden zu können.

Die derzeit verfügbaren Meßverfahren zur quantitativen Bestimmung von ADMA und SDMA in Plasma, Serum, Urin und anderen biologischen Flüssigkeiten (Gewebsextrakten, Zellkulturüberständen u.a.) basieren allesamt auf dem chemischen Nachweisverfahren der Hochdruck-Flüssigkeitschromatografie (HPLC). Verschiedene Modifikationen von HPLC-Methoden werden hierzu derzeit eingesetzt. Diese Methoden sind jedoch sehr zeit- und personalintensiv, teuer, und somit für die klinische Routinediagnostik nicht geeignet.

Aufgabe der vorliegenden Erfindung ist es, eine Wahrscheinlichkeit des zukünftigen Fortschreitens von Erkrankungen nachzuweisen, die mit einer Störung des NO-Stoffwechsels einhergehen.

Diese Aufgabe wird erfindungsgemäß dadurch gelöst, daß ein Gehalt von endogenen Methyl-Argininen in biologischen Flüssigkeiten bestimmt wird.

Trotz der sehr umfänglichen Bestimmung verschiedenster bisher bekannter kardiovaskulärer Risikofaktoren ist bei einem Anteil von Patienten trotz Fehlens solcher bisher bekannter Risikofaktoren ein Fortschreiten der genannten Erkrankungen nachzuweisen. Die Erfindung weist den Vorteil auf, daß zumindest ein Teil dieser Fälle durch das Vorliegen erhöhter Konzentrationen der endogenen Methyl-Arginine erklärt werden kann und somit eine verbesserte Prognose über den Krankheitsverlauf abgegeben werden kann.

Die bisher eingesetzten Nachweisverfahren für ADMA und SDMA basieren auf dem Prinzip der HPLC, was sie sehr aufwendig und teuer macht, so daß diese Verfahren nicht umfassend in klinischen Alltag eingesetzt werden können, sondern spezialisierten Forschungslabors vorbehalten bleiben. Eine weitere Aufgabe der Erfindung ist es daher, ein Verfahren vorzulegen, mit dessen Hilfe einfach, kostengünstig und universell klinisch nutzbar die endogenen Methyl-Arginine ADMA und SDMA quantitativ in biologischen Flüssigkeiten bestimmt werden können.

Diese Aufgabe wird erfindungsgemäß dadurch gelöst, daß zur Bestimmung des Gehaltes von endogenen Methyl-Argininen in einer biologischen Flüssigkeit auf diese mit Antikörpern eingewirkt wird.

Die Bestimmung der ADMA- und SDMA-Konzentrationen mittels immunochemischer Verfahren (d.h. unter Verwendung von monoklonalen oder polyklonalen Antikörpern) hat erfindungsgemäß wesentliche Vorteile:

1. Die Messung der ADMA- und SDMA-Konzentrationen mittels Verwendung antikörperbasierter Verfahren muß nicht in hochspezialisierten HPLC-Labors durchgeführt werden. Immunochemische Nachweisverfahren wie Radioimmunoassays, Enzymimmunoassays etc. sind in den meisten klinisch-chemischen Labors routinemäßig verfügbar.

2. Die Messung mit immunochemischen Nachweisverfahren dauert weniger lange als die Messung mittels HPLC. Für letzteres Verfahren ist eine aufwendige Probenextraktion aus der jeweiligen Matrix (Plasma, Serum, Urin o.a.) erforderlich, damit die Messung per HPLC erfolgen kann. Das rasche Vorliegen des Meßwertes ist aber für die Verwendung des ADMA- bzw. SDMA-Nachweises im klinischen Alltag unabweisbar notwendig, damit dieser Parameter als Krankheits-Marker breite Akzeptanz finden kann.

3. Immunochemische Nachweisverfahren können neben der klinischen Routine auch in experimentellen Anwendungen zum Einsatz kommen (z.B. Immunohistochemie, Immunozytochemie, Immunoblotting).

4. Entsprechend der vorliegenden Erfindung ist der diagnostische Nachweis von ADMA bzw. SDMA geeignet, um prospektiv über das Erkrankungs- oder Todes-Risiko eines Patienten Aufschluß zu erlangen ("Risikofaktor").

Die Erzeugung monoklonaler Antikörper erfolgt nach allgemein bekannten immunologischen Verfahren durch Fusion von immunkompetenten Zellen sensibilisierter Versuchstiere mit Myelomzellen und Selektion der die spezifischen Antikörper produzierenden Zellklone mittels Standardverfahren. Die Erzeugung polyklonaler Antikörper erfolgt mittels Gewinnung von Immunserum von immunisierten Versuchstieren nach allgemein bekannten Verfahren. Die Produktion monoklonaler Antikörper gegen ADMA und SDMA umfaßt die Kultivierung der Antikörper-produzierenden Zellklone, die Gewinnung des Antikörper-enthaltenden konditionierten Mediums, sowie die Abfüllung und den Vertrieb der Antikörperlösungen. Die Nutzung der Antikörper umfaßt ihren Einsatz zu diagnostischen und wissenschaftlichen Zwecken, als Antikörper-Suspension oder als

Bestandteil eines diagnostischen Kits, in den Bereichen der klinischen Medizin, der experimentellen Medizin, der Veterinärmedizin, der Biologie und anderer Biowissenschaften.

Die nachstehenden Beispiele erläutern die Erfindung:

Beispiel 1

225 Patienten mit chronischer Niereninsuffizienz wurden in eine klinische Studie aufgenommen. Zu Beginn der Studie wurde jedem Patienten eine Blutprobe entnommen, in der die Konzentrationen von ADMA und SDMA sowie von L-Arginin bestimmt wurden. Die Patienten wurden über eine mittlere Beobachtungsdauer von 26 Monaten (1 – 35 Monaten) nachbeobachtet. Während dieser Zeit erlitten 57 Patienten ein kardiovaskuläres Krankheitsereignis (Myokardinfarkt, Schlaganfall, periphere Durchblutungsstörung oder kardial bedingter Tod). Patienten mit einem solchen kardiovaskulären Krankheitsereignis hatten zu Beginn der Studie signifikant höhere ADMA-Konzentrationen gehabt (Median 3,2 µmol/L) als Patienten ohne solches Ereignis (Median 2,2 µmol/L). ADMA erwies sich in der statistischen Analyse als ein unabhängiger Prädiktor kardiovaskulärer Krankheitsereignisse. Wurden die Patienten anhand der zu Beginn der Studie gemessenen ADMA-Plasmakonzuentrationen in Gruppen eingeteilt (ADMA < 50. Perzentile, 51. – 70. Perzentile, 71. – 90. Perzentile, und >90. Perzentile), so ergab sich eine klare Zunahme der Inzidenz kardiovaskulärer Todesfälle mit steigender ADMA-Konzentration (Abbildung 1).

Beispiel 2

Bei 225 Patienten, bei denen in einer zu Beginn der Studie abgenommenen Blutprobe die ADMA-Konzentration bestimmt worden war, traten im Verlauf einer im Mittel 26 Monate dauernden Nachbeobachtungsphase insgesamt 57 Todesfälle aufgrund verschiedenster Ursachen auf. Patienten, die im Verlauf der Studie verstarben, hatten zu Beginn signifikant höhere ADMA-Konzentrationen aufgewiesen (Median 3,5 µmol/L) als Patienten, die überlebten (2,3 µmol/L). ADMA erwies sich als ein von anderen Risikofaktoren unabhängiger Prädiktor des Überlebens. Wurden die Patienten anhand der zu Beginn der Studie gemessenen ADMA-

Plasmakonzentrationen in Gruppen eingeteilt (ADMA < 50. Perzentile, 51. – 70. Perzentile, 71. – 90. Perzentile, und >90. Perzentile), so ergab sich eine klare Zunahme der Gesamtmortalität mit steigender ADMA-Konzentration (Abbildung 2).

Beispiel 3

Bei 90 Patienten wurde in einer Plasmaprobe die ADMA-Konzentration bestimmt. Anschließend wurde bei diesen Patienten mittels hochauflösender Ultraschalluntersuchung die Wanddicke der Halsschlagader (A. carotis) vermessen. ADMA war ein unabhängiger Einflußfaktor für die Intima/Media-Dicke der A. carotis (Abbildung 3), für die luminale Querschnittsfläche, und für den Schweregrad der arteriosklerotischen Läsionen in der A. carotis.

Beispiel 4

Bei 45 Patienten mit chronischer Herzinsuffizienz wurde eine Blutprobe entnommen. Die Messung der ADMA-Konzentration ergab einen mittleren Wert von 4,1 ± 0,8 µmol/l, im Vergleich zu 1,0 ± 0,1 µmol/l bei gesunden Kontrollpersonen. Patienten mit höheren ADMA-Konzentrationen hatten eine geringere NO-vermittelte Gefäßdilatation (R = -0.79) und eine geringere maximale Sauerstoffaufnahme unter körperlicher Belastung (R = -0.81; Abbildung 2a). Die Gabe von L-Arginin führte zu einer signifikanten Verbesserung der NO-vermittelten Gefäßdilatation; das Ausmaß dieser Verbesserung durch L-Arginin war umso größer, je höher die ADMA-Konzentration beim jeweiligen Patienten war (R = 0.74; Abbildung 2b).

Patentansprüche

1. Verfahren zum Nachweis einer Wahrscheinlichkeit des zukünftigen Auftretens oder Fortschreitens von Erkrankungen, die mit einer Störung des NO-Stoffwechsels einhergehen, dadurch gekennzeichnet, dass ein Gehalt von endogenen Methyl-Argininen in einer biologischen Flüssigkeit nachgewiesen wird.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Erkrankung eine koronare Herzkrankheit ist.

3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Erkrankung eine chronische Herzinsuffizienz ist.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Erkrankung eine erektile Dysfunktion ist.

5. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Erkrankung eine periphere arterielle Durchblutungsstörung ist.

6. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Erkrankung eine chronische Niereninsuffizienz ist.

7. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Erkrankung eine zerebrale ischämische Erkrankung ist.

8. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Erkrankung ein Diabetes mellitus ist.

9. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Erkrankung eine Präeklampsie ist.

10. Verfahren nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, daß festgestellt wird, wie hoch in der biologischen Flüssigkeit eine Konzentration von ADMA ist.

11. Verfahren nach einem der Ansprüche 1 bis 10, dadurch gekennzeichnet, daß festgestellt wird, wie hoch in der biologischen Flüssigkeit ein Verhältnis der Konzentrationen von L-Arginin zu ADMA ist.

12. Verfahren nach einem der Ansprüche 1 bis 10, dadurch gekennzeichnet, daß festgestellt wird, wie hoch in der biologischen Flüssigkeit eine Konzentration von SDMA ist.

13. Verfahren nach einem der Ansprüche 1 bis 12, dadurch gekennzeichnet, daß festgestellt wird, wie hoch in der biologischen Flüssigkeit ein Verhältnis der Konzentrationen von L-Arginin zu SDMA ist.

14. Verfahren nach einem der Ansprüche 1 bis 13, dadurch gekennzeichnet, daß festgestellt wird, wie hoch in der biologischen Flüssigkeit ein Verhältnis der Konzentrationen von ADMA zu SDMA ist.

15. Verfahren nach einem der Ansprüche 1 bis 14, dadurch gekennzeichnet, daß zur Bestimmung des Gehaltes von endogenen Methyl-Argininen in einer biologischen Flüssigkeit auf diese mit Antikörpern eingewirkt wird.

16. Verfahren nach Anspruch 15, dadurch gekennzeichnet, daß mit monoklonalen Antikörpern auf die das endogene Methyl-Arginin enthaltende biologische Flüssigkeit eingewirkt wird.

17. Verfahren nach Anspruch 15 oder 16, dadurch gekennzeichnet, daß mit polyklonalen Antikörpern auf die das endogene Methyl-Arginin enthaltende biologische Flüssigkeit eingewirkt wird.

18. Verfahren nach Anspruch 17, dadurch gekennzeichnet, daß die monoklonalen Antikörper durch Kultivierung der Antikörper-produzierenden Zellklone und Gewinnung einer die Antikörper enthaltenden Lösung gewonnen werden.

19. Verfahren nach Anspruch 17, dadurch gekennzeichnet, daß die polyklonalen Antikörper durch Gewinnung von Immunserum von immunisierten Versuchstieren erzeugt werden.

20. Mittel zur Durchführung der Verfahren 1 bis 19, dadurch gekennzeichnet, daß die biologische Flüssigkeit aus Plasma besteht.

21. Mittel zur Durchführung der Verfahren 1 bis 19, dadurch gekennzeichnet, daß die biologische Flüssigkeit aus Serum besteht.

22. Mittel zur Durchführung der Verfahren 1 bis 19, dadurch gekennzeichnet, daß die biologische Flüssigkeit aus Urin besteht.

23. Mittel zur Durchführung der Verfahren 1 bis 19, dadurch gekennzeichnet, daß die biologische Flüssigkeit aus Gewebsextrakten besteht.

24. Mittel zur Durchführung der Verfahren 1 bis 19, dadurch gekennzeichnet, daß die biologische Flüssigkeit aus histologischen Präparaten besteht.

25. Mittel zur Durchführung der Verfahren 1 bis 19, dadurch gekennzeichnet, daß die biologische Flüssigkeit aus zytologischen Präparaten besteht.

Abbildung 1

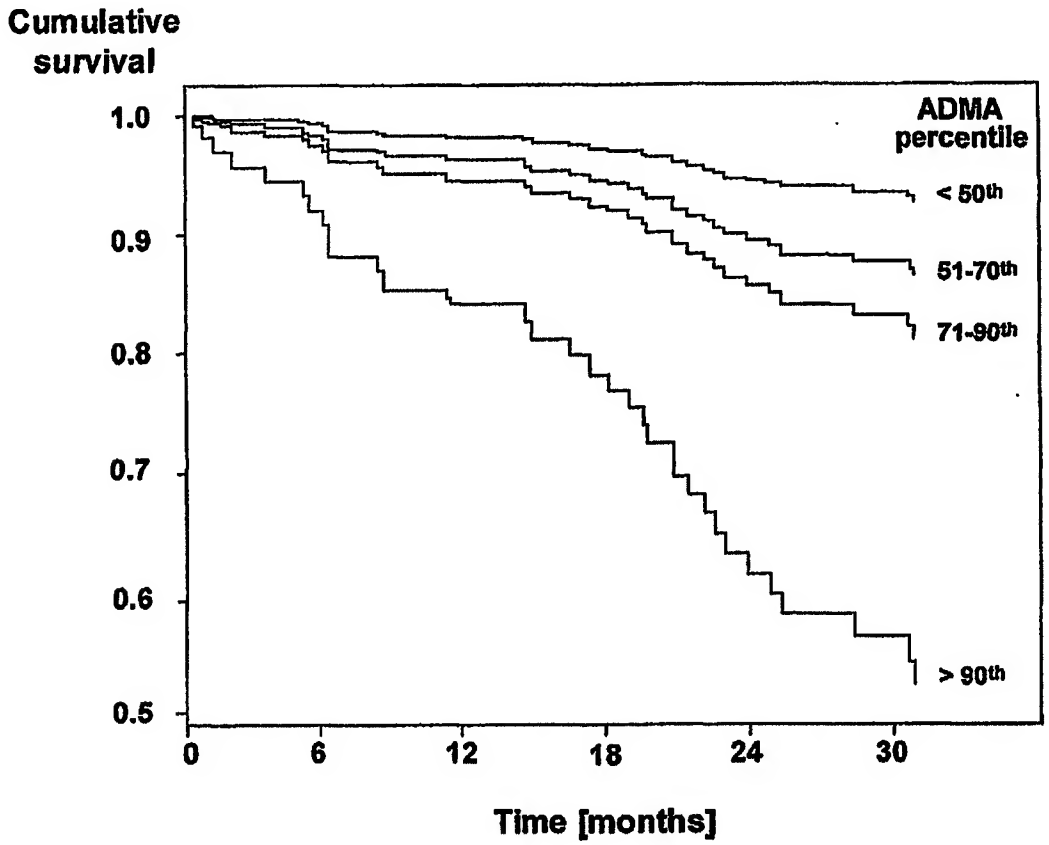
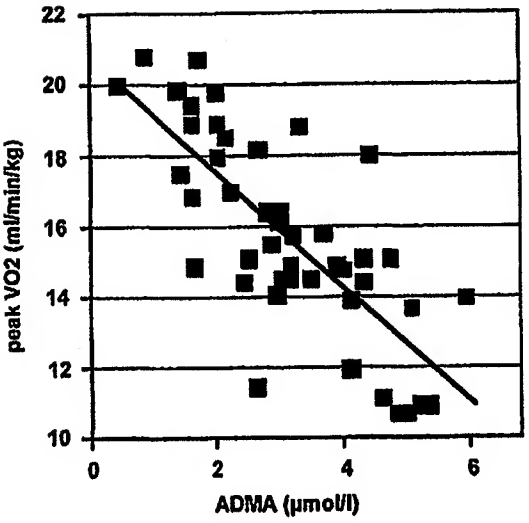

Cumulative survival
1.0
0.9
0.8
0.7
0.6
0.5
0
6
12
18
24
30
Time [months]
ADMA percentile
< 50th
51-70th
71-90th
> 90th

Abbildung 2

a.



b.

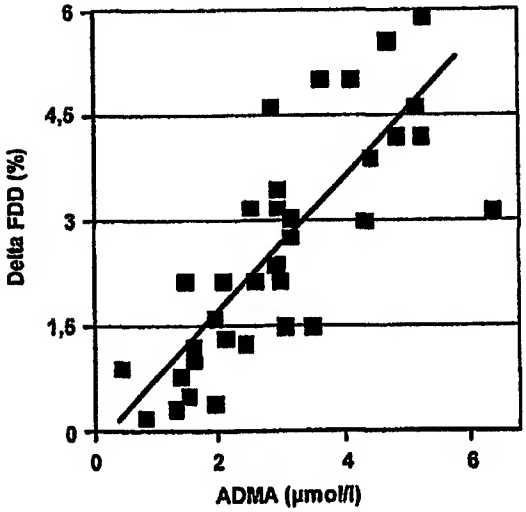


EXHIBIT 4

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/553,462	05/05/2006	Makrina Savvidou	HO-P03236US0	8934

29053 7590 12/09/2010
FULBRIGHT & JAWORSKI L.L.P
2200 ROSS AVENUE
SUITE 2800
DALLAS, TX 75201-2784

EXAMINER	
SINGH, ANOOP KUMAR	

ART UNIT	PAPER NUMBER
1632	

NOTIFICATION DATE	DELIVERY MODE
12/09/2010	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

doipdocket@fulbright.com

Office Action Summary	Application No.	Applicant(s)	
	10/553,462	SAVVIDOU ET AL.	
	Examiner	Art Unit	
	ANOOP SINGH	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *22 September 2010*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,6-8 and 11* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1, 6-8 and 11* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Applicants' amendments and arguments filed September 22, 2010 has been received and entered. Claims 1, 6-8 and 11 are pending.

Election/Restrictions

Applicant's election without traverse of claims 1-2 and 4-11 in the reply filed on August 31, 2007 was acknowledged.

Claims 1, 6-8 and 11 are under consideration.

Maintained -Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1, 6-7 and 8 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Holden et al (Am J Obstet Gynecol. 1998; 178(3):551-6, art of record), Ellis et al (Acta Obstr. Gynecol Scand 2001, 80, 602-608, IDS) and Boger (WO 2002/14873, 2/21/2002, IDS) for the reasons of record.

Claims are directed a method of determining that a pregnant woman is at risk of developing pre-eclampsia or that her fetus is at risk of developing intrauterine growth restriction (IUGR), which method comprises: (a) measuring asymmetric dimethylarginine (ADMA) in a plasma sample taken from a pregnant woman at a stage of pregnancy from 23 to 25 weeks gestation; and (b) determining that the woman is at risk of developing pre-eclampsia or her fetus is at risk of developing IUGR if the level of ADMA in the plasma sample is greater than 1.5 gmol/L. Claim 6 limits the method of claims 1, wherein determining that the woman is at risk of developing pre-eclampsia or determining that her fetus is at risk of developing IUGR comprises determining that the woman's ADMA level is at least 3 times the normal pregnancy level.

Holden et al teach a method comprising (a) measuring asymmetric dimethylarginine (ADMA) in a plasma sample taken from a pregnant woman at different stage of pregnancy; and (b) determining the level of ADMA in the plasma sample. It is noted that Holden et al also

determined the level of ADMA to be around 0.52 μmol/L to 1.17 μmol/L during second trimester. This would meet the claim limitation of measuring pregnancy at different stage of pregnancy (including 23-25 weeks) that is embraced by the teaching of Holden (see page 553, Figure 1 B). It is further disclosed that pregnant woman have pre-eclampsia if ADMA in the plasma sample is 1.25 gmol/L (see figure 1A). Therefore, any ADMA level greater than 0.75 μmol/L would also have PE meeting the limitation of the claim. It is further noted that Holden et al conclude that during later stage of pregnancy circulating concentrations increase and, when pregnancy is complicated by preeclampsia. Thus, method of Holden is primarily directed to study the role for ADMA in the changes in blood pressure seen in both normal and preeclampsia pregnancy (see abstract and page 555, col. 1, para. 4). While Holden et al teach a method of (a) measuring ADMA level at least in pregnant women and reported (d) determining that woman has pre-eclampsia if ADMA in the plasma sample is greater than1.17 gmol/L, but differ from claimed invention by not measuring the ADMA level in women at 23 to 25 weeks gestation.

However, such was disclosed by Ellis et al, teach a method comprising (a) measuring asymmetric dimethylarginine (ADMA) in a plasma sample taken from a pregnant woman at different stage of pregnancy including 24-32 weeks gestation; and (b) determining the level of ADMA in the plasma sample (limitation of claim 1). It is disclosed that plasma concentrations of asymmetric dimethylarginine (ADMA) are significantly elevated both in mild preeclarnpsia during pregnancy from 24 to 32 weeks gestation that includes 24 and 25 weeks gestation (see abstract, figure 1 and 2). It is relevant to point out that Ellis teaches measuring ADMA level in plasma of pregnant woman at stage 24-25 weeks gestation and reported that pregnant woman have pre-eclampsia if ADMA in the plasma sample is greater than 0.75μmol/L (see figure 1), which is significantly higher than normal level ($p<0.04$) (see figure 1). Ellis et al further contemplate studying ADMA and SDMA level early in the pregnancy in order to ascertain if levels rise early enough to predict preeclampsia (see page 607, col. 1, para. 1). While Ellis et al teach a method of measuring ADMA level in women at 24 to 32 weeks gestation having preeclampsia, but differ from claimed invention by not measuring the ADMA level in non pre screened pregnant women.

Boger et al cure the deficiency by teaching a method of detecting the risk of developing a disease including pre-eclampsia that is associated with NO metabolism by (a) measuring the level of ADMA and SDMA (see claims 1 and 9). Boger et al also disclose that preeclampsia is a disease of the NO metabolism leads to constriction of arteries which induces high blood pressure in the mother and poses a risk to the unborn child due to reduced placental perfusion (see page 2) With respect to claim 7 and 8, Boger et al contemplate measuring the ratio of ADMA to SDMA in the plasma of the patient (see claim 14, 20 and 21). It is also disclosed that subject suffering from chronic conditions (CHF, example 4) show ADMA concentration of 4.1 μM/L as compared to 1.0 μM/L in normal subject.

It would have been obvious for one of ordinary skill in the art at the time of invention to modify the method of Holden of measuring the ADMA level in detecting the risk of developing a disease including pre-eclampsia in the mother due to reduced placental perfusion as disclosed by Boger using the known method disclosed by Holden and Ellis. It would have been *prima facie* obvious to one of ordinary skill in the art to combine the known methods of Holden, Ellis and Boger to measure the ADMA level in a pregnant women at a stage of pregnancy comprising 23-25 and determine the level of ADMA to detect the risk of developing of pre-eclampsia

particularly since both Holden and Ellis generally embraced the potential of measuring ADMA level to determine the risk of developing pre-eclampsia. Other limitations of measuring ADMA level that is at least 3 times or ADMA/SDMA level 5 times than the normal pregnancy level would be implicit in the method taught by the combination of references and therefore would be obvious variables when measuring the level of ADMA or SDMA in pregnant women predisposed to develop PE as disclosed by Ellis. One who would have practiced the invention would have had reasonable expectation of success since Ellis and Holden both taught method to measure ADMA level in the plasma of subject to determine if the subject is at risk of developing PE, while combining the teaching Holden, Ellis with Boger would have resulted in a determining that woman is at risk of developing PE if the ADMA level is greater then normal control as suggested by Ellis.

Therefore, the claimed invention would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention.

Claims 1 and 11 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Holden et al (Am J Obstet Gynecol. 1998; 178(3):551-6, art of record), Ellis et al (Acta. Obstr. Gynecol. Scand. 2001: 80, 602-608, IDS) and Boger (WO 2002/14873, 2/21/2002, IDS) as applied to claims 1, 6-8 above, and further in view of Albaiges et al (Obstet Gynecol 2000;96:559-64, IDS) for the reasons of record.

The teachings of Holden et al, Ellis et al and Boger were described above and relied in same manner here. The combination of art teach a method of determining that a pregnant woman is at risk of developing pre-eclampsia or that her fetus is at risk of developing intrauterine growth restriction (IUGR), which method comprises measuring asymmetric dimethylarginine (ADMA) in a plasma sample taken from a pregnant woman at a stage of pregnancy from 23 to 25 weeks gestation, but differ from claimed invention by not disclosing use of Doppler waveform analysis of uterine arteries and/or flow mediated dilatation of the brachial artery in the women.

However, use of Doppler wave form to predict PE in pregnant women was known and routinely used by one of ordinary skill in the art. For instance, Albaiges et al discloses color doppler of uterine artery imaging of women with singleton pregnancies at 23 weeks to determine bilateral uterine artery notches, left and right uterine artery pulsatility indices (PI) for predicting preeclampsia and delivery of small-for-gestational-age infants (See abstract).

Therefore, it would have been *prima facie* obvious for a person of ordinary skill in the art seeking to predict risk of developing PE would combine the respective teachings of Holden et al, Ellis et al and Boger by modifying the method to further to further include Doppler waveform analysis of uterine arteries to determine if a pregnant woman is at risk of developing PE as disclosed by Albaiges et al, with a reasonable expectation of success. A person of skill in the art would have been motivated to modify the method by further conducting Doppler analysis as disclosed by Albaiges et al, as a matter of design choice, said design choice amounting to combining prior art method directed to diagnose same condition (PE) according to known methods to yield predictable results. One of ordinary skill in the art would be motivated to use color Doppler for diagnosis of PE because Albaiges et al teaches successful use of color doppler

of uterine artery imaging of women with singleton pregnancies at 23 weeks predict preeclampsia in women risk of developing PE (supra) . One of skill in the art would have been expected to have a reasonable expectation of success in determining if a pregnant woman is at risk of developing pre-eclampsia or IUGR by measuring ADMA and color Doppler imaging because the art teaches the successful diagnosis of PE by measuring plasma ADMA and use of Doppler waveform analysis of uterine arteries. It should be noted that the *KSR* case forecloses the argument that a specific teaching, suggestion, or motivation is required to support a finding of obviousness See the recent Board decision *Ex parte Smith*, --USPQ2d--, slip op. at 20, (Bd. Pat. App. & Interf. June 25, 2007) (citing *KSR*, 82 USPQ2d at 1396) (available at http: www. uspto.gov/web/offices/dcom/bpai/prec/fd071925.pdf).

Response to arguments

Applicants disagree with the rejection of claim 1 over Holden et al in view of Ellis and Boger, arguing that Holden describes ADMA levels of pre-eclamptic patients during the third trimester that is outside of the 23-25 week. Applicant assert that a a measurement 1.17 mmol/L at 23-25 weeks would not trigger a determination that a woman is at risk of developing pre-eclampsia or that her fetus is at risk of developing IUGR. Applicants further argue that Ellis describes testing patients to determine ADMA levels at a stage of pregnancy of 24-32 weeks. The range of ADMA levels of pre-eclamptic patients was determined to be in the range of 0.4-.1 μmol.L). Applicant assert that Ellis does not disclose an ADMA level of greater than 1.5 μmol/L. Applicants argue that the levels observed by Ellis are lower than the level of 1.17 μmol/L observed by Holden (see page 4, para. 3-4 of the arguments). Applicants further argue that Boger do not cure the deficiency and thus conclude that none of the references suggests that a level of 1.5 μmol/L or greater at a stage of pregnancy from 23 to 25 weeks would be indicative of preeclampsia. Applicants' arguments have been fully considered, but are not found persuasive.

In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.,* 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986). Applicants have further engaged in selective reading of the teachings of Holden et al in view of Ellis. to formulate the grounds for teaching away. It should be noted that the ultimate goal of measuring ADMA level in plasma of a pregnant woman is to suggest that a changes in the circulating concentration of ADMA is

important in the pathophysiologic mechanisms of preeclampsia through a reduction in nitric oxide synthesis (see Holden et al, page 552, col. 1, last para).

As previously indicated, Holden et al in describing method disclose (a) measuring asymmetric dimethylarginine (ADMA) in a plasma sample taken from a pregnant woman at different stage of pregnancy, and (b) determining the level of ADMA in the plasma sample. It is noted that Holden et al also determined the level of ADMA to be around 0.52 μmol/L to 1.17 μmol/L during second trimester (supra), but differ from claimed invention by not measuring the ADMA level in women at 23 to 25 weeks gestation. However, measuring level of plasma ADMA in pregnant woman at 24-34 weeks of gestation and determining if the subject woman has higher level of ADMA (>0.8μmol/L, Fig. 1 Ellis) suggesting risk of mild pre-eclampsia was known (see Ellis et al Figure 1). In view of foregoing teachings in prior art, one of ordinary skill in the art would conclude that a higher plasma level of ADMA ((>0.8μmol/L, Fig. 1 Ellis) in a pregnant woman (24-32weeks gestation) would put the woman at risk of developing pre-eclampsia. To the extent that Ellis. describe the measuring ADMA level in a plasma taken from a pregnant woman at a stage 24-34 weeks gestation and determining that woman is risk of developing PE if ADMA level is greater than 0.8, the rejection is applicable to the instant case. Applicants' selective reading of Holden et al. ignores the teachings of the reference of Ellis. There is no requirement for Holden et al. to teach that which is clearly taught by Ellis et al. It should be noted that determining that the woman is at risk of developing mild PE if the plasma level greater that 0.8μmol/l as exemplified in Ellis would necessarily mean that ADMA level any higher than 0.8μmol/L such as 1.5 μmol/L must also put the woman at risk of developing pre-eclampsia (PE) (emphasis added).

Applicant should further note that prior art summarized by the reference of Boger et al is applied to the extent it teaches method of detecting the risk of developing a disease including pre-eclampsia that is associated with NO metabolism by (a) measuring the level of ADMA and SDMA (see claims 1 and 9). Boger et al also disclose that preeclampsia is a disease of the NO metabolism leads to constriction of arteries which induces high blood pressure in the mother and poses a risk to the unborn child due to reduced placental perfusion (see page 2) It is also disclosed that subject suffering from chronic conditions (CHF, example 4) show ADMA

concentration of 4.1 μM/L as compared to 1.0 μM/L in normal subject. Therefore, it is apparent from the teaching of Boger that higher level of ADAM could also determined if the pregnant woman has underlying cardiac or other conditions (CHF, smoking and obesity) that are known to further increase plasma ADMA level. Thus, contrary to applicants' arguments plasma ADMA level in the pregnant woman could vary depending upon various under lying conditions that are known in prior art. To the extent prior art teaches determining plasma level greater than 0.8μmol/liter predisposes woman of developing PE, it must necessarily mean that an ADMA level any higher than 0.8μmol/L such as 1.5 μmol/Las claimed in the instant application must also put the woman at risk of developing PE. Therefore, teaching of Holden, Ellis and Boger would be been *prima facie* obvious to one of ordinary skill in the art at the time of the invention.

Therefore, in view of the fact patterns of the instant case, and the ground of rejection outlined by the examiner, applicants' arguments are not compelling and do not overcome the rejection of record.

On pages 5, last para and page 5 , first para of the arguments, Applicant re-iterates and rely on their previous arguments that have been discussed in preceding section . The arguments are substantially the same as those addressed in the foregoing response.

Should applicants provide evidence pertinent to secondary consideration to obviousness relating to criticality of five times or more ADMA/SDMA level in a pregnant woman at a stage 23 to 25 weeks gestation predisposes said woman to PE, instant obviousness may be overcome, pending further consideration.

Conclusion

No claims allowed.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period

will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ANOOP SINGH whose telephone number is (571)272-3306. The examiner can normally be reached on 9:00AM-5:30PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Peter Paras can be reached on (571) 272- 4517. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Anoop Singh/
Examiner, Art Unit 1632

EXHIBIT 5

One-Stage Screening for Pregnancy Complications by Color Doppler Assessment of the Uterine Arteries at 23 Weeks' Gestation

GERARD ALBAIGES, MD, HANNAH MISSFELDER-LOBOS, MD, CHRISTOPH LEES, MD, MAURO PARRA, MD, AND KYPROS H. NICOLAIDES, MD

Objective: To estimate the value of screening for preeclampsia and fetal growth restriction by performing color Doppler assessment of uterine arteries at 23 weeks' gestation in predicting adverse pregnancy outcome.

Methods: Women with singleton pregnancies who attended routine ultrasonography at 23 weeks had color Doppler uterine artery imaging. Bilateral uterine artery notches were noted and left and right uterine artery pulsatility indices (PI) were measured. A mean PI of more than 1.45 was considered increased. Screening characteristics for predicting preeclampsia and delivery of small-for-gestational-age infants were calculated.

Results: Of 1757 pregnancies, increased PI was present in 89 (5.1%) and bilateral notches were noted in 77 (4.4%). Twenty-three of 65 women (35.3%; 95% confidence interval [CI] 23.9, 48.2) had increased PI and later developed preeclampsia, and 8 of 10 (80%; 95% CI 44.4, 97.5) with preeclampsia required delivery before 34 weeks. The respective values for women with bilateral notches were 21 of 65 (32.3%; 95% CI 21.2, 45.1) and 8 of 10 (80%; 95% CI 44.4, 97.5). The sensitivity of increased PI was 30 of 143 (21%; 95% CI 14.6, 28.6) for delivery of an infant with birth weight below the tenth percentile and 7 of 10 (70% 95% CI 34.8,93.3) for birth weight below the tenth percentile delivered before 34 weeks. The respective values for bilateral notches were 19 of 143 (13.3%; 95% CI 8.2, 20) and 5 of 10 (50%; 95% CI 18.7, 81.3).

Conclusion: A one-stage color Doppler screening program at 23 weeks identified most women who subsequently developed serious complications of impaired placentation associated with delivery before 34 weeks. The screening results were similar when the high-risk group was defined as women with increased PI or bilateral notches. (Obstet Gynecol 2000;96:559–64. © 2000 by The American College of Obstetricians and Gynecologists.)

From Harris Birthright Research Centre for Fetal Medicine, King's College Hospital, London, United Kingdom.

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Preeclampsia, fetal growth restriction (FGR), placental abruption, and some cases of fetal death during the latter half of pregnancy are believed to result from impaired placentation in early gestation.[1] Deficient placentation is characterized by inadequate trophoblast invasion into the maternal spiral arteries and failure to develop low-resistance uteroplacental circulation. In the past 20 years, Doppler ultrasonographic studies of uteroplacental circulation have shown that high impedance to flow is associated with subsequent preeclampsia, FGR, and related complications.[2]

In our hospital, uterine artery Doppler assessment used to be an integral part of 20-week scanning that was offered to all pregnant women. Women with abnormal Doppler results were examined again at 24 weeks, and those with persistently abnormal results were followed in a high-risk clinic. The data from that two-stage approach were reported[3,4] and variations on the design were described.[5,6] Harrington et al[4] found bilateral early diastolic notches in about 3.9% of the population, a group that contained about 54.5% of women who subsequently developed preeclampsia and 21.8% of those who delivered infants with birth weights below the tenth percentile for gestation.

Our policy on the second-trimester scan changed recently. The scan is done at 23 weeks and color Doppler is used in all cases to examine uterine arteries. This study examined the performance of one-stage color Doppler ultrasonography in predicting adverse pregnancy outcomes.

Methods

Women who had routine antenatal care at King's College Hospital, London, an inner-city teaching hospital, had color Doppler examination of the uterine arteries at 23 weeks' gestation (Acuson Aspen, Acuson Co., Moun-

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PII S0029-7844(00)00946-7

tain View, CA or Aloka SSD-1700, Aloka Co., Tokyo, Japan). The right and left uterine arteries were identified at the apparent crossover with the external iliac arteries, and pulsed-wave Doppler was used to obtain waveforms. When three similar consecutive waveforms were obtained, an early diastolic notch was noted, the pulsatility index (PI) was measured, and the mean PI of the two vessels was calculated.

Women with normal uterine artery Doppler received routine antenatal care. Those with bilateral uterine artery notches or those with mean PIs of 1.45 or higher constituted the screen-positive group and were followed in a high-risk clinic starting at 24 weeks' gestation. A PI of 1.45 was chosen because it corresponds to the 95th percentile of the reference range for our population at that gestation.

Adverse outcomes were defined as preeclampsia, and birth weight less than the tenth and third percentiles for gestation[7] before 34 weeks. Other adverse outcomes were fetal death and placental abruption (defined as vaginal bleeding leading to emergency delivery and evidence of retroplacental clot at delivery). Preeclampsia was defined by blood pressure of 140/90 mmHg or greater on two occasions more than 2 hours apart, with proteinuria (minimum of 300 mg per 24 hours or dipstick testing of 300 mg/L).

During the study (May through November 1998), 1941 consecutive women with singleton pregnancies who attended the ultrasonography unit had uterine artery Doppler examinations at 22 to 25 (mean, 23) weeks' gestation. Doppler findings were recorded in a computer patient database, and thermal waveform images were retained. Complete demographic and outcome data were available for 1757 (90.6%) women. No women were subsequently excluded from the analysis. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated by using the Statistical Package for Social Sciences, version 6 (SPSS, Inc., Chicago, IL). Receiver-operating characteristic (ROC) curves for mean PI relating to adverse outcomes were generated by using a logarithmic trendline in the Microsoft Excel 97 software package (Microsoft, Inc., Redmond, WA).

Results

Complete demographic and outcome data were available for 1757 women (Tables 1 and 2). Their mean age was 30 years (range, 18 to 44 years). Abnormal Doppler results were present in 128 women (7.3%), including 77 (4.4%) with bilateral notches, 89 (5.1%) with mean PIs above 1.45, and 38 (2.2%) with high PIs and notches. In the group with missing outcomes, the proportion with high PIs or bilateral notches was 9 of 184 (5.1%), which was similar to that in patients with complete follow-up.

Table 1. Demographic Characteristics of the Screened Population

	No. of patients (%)
Parity	
Nulliparous	934 (52.6)
Multiparous	823 (47.4)
Smoking	
>5/day	273 (16.1)
Nonsmoker	1484 (83.9)
Ethnicity	
White	792 (45.1)
Black	711 (40.4)
Asian	117 (6.7)
Other	137 (7.8)

Sensitivity, specificity, positive and negative predictive values, and relative risks of adverse outcomes according to abnormal Doppler results are shown in Tables 3 (bilateral uterine artery notches or mean PI above 1.45), 4 (bilateral notches), 5 (high mean PI), and 6 (high mean PI and bilateral notches). Receiver-operating characteristic curves are shown for mean PIs relating to preeclampsia, preeclampsia requiring delivery before 34 weeks, delivery of infants with birth weights less than the tenth percentile, and less than the third percentile overall and before 34 weeks (Figures 1 and 2).

Discussion

The one-stage color Doppler uterine artery screening program at 23 weeks' gestation classified about 5% of the population as high-risk. That group contained about 90% of women who developed preeclampsia that required delivery before 34 weeks, 70% of those whose infants had birth weights less than the tenth percentile

Table 2. Adverse Outcomes in the Screened Population (n = 1757)

	No. of patients (%)
Preeclampsia	65 (3.70)
Preeclampsia, delivery before 34 wk	10 (0.57)
Birth weight below tenth percentile	143 (8.14)
Birth weight below third percentile	52 (2.96)
Birth weight below tenth percentile, delivery before 34 wk	10 (0.57)
Birth weight below third percentile, delivery before 34 wk	9 (0.51)
Fetal death	6 (0.34)
Placental abruption	10 (0.57)

Table 3. Screening Characteristics for Bilateral Notches or Mean Pulsatility Index Above 1.45

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Relative risk
Preeclampsia (n = 65)	44.6 (32.2, 57.4)	94.1 (92.9, 95.2)	22.6 (15.6, 30.7)	97.7 (97, 98.4)	10.2
Preeclampsia, delivery before 34 wk (n = 10)	90 (55.5, 99.7)	93 (91.7, 94.2)	7 (3.2, 12.6)	99.9 (99.7, 100)	114.5
Birth weight below tenth percentile (n = 143)	22.3 (15.8, 30.1)	94 (92.7, 95.2)	25 (17.6, 33.2)	93.1 (91.8, 94.4)	3.7
Birth weight below third percentile (n = 52)	30.2 (18.7, 45.1)	93.4 (92.1, 94.5)	12.5 (7.3, 19.4)	97.8 (96.9, 98.4)	5.5
Birth weight below tenth percentile, delivery before 34 wk (n = 10)	70 (34.8, 93.3)	93 (91.7, 94.2)	5.5 (2.2, 10.9)	99.9 (99.5, 100)	29.7
Birth weight below third percentile, delivery before 34 wk (n = 9)	77.8 (40, 97.2)	93.1 (91.7, 94.2)	5.5 (2.2, 10.9)	99.9 (99.6, 100)	44.7
Fetal death (n = 6)	83.3 (35.9, 99.6)	92.8 (91.5, 94)	3.9 (1.3, 8.7)	99.9 (99.7, 100)	63.6
Placental abruption (n = 10)	50 (18.8, 81.3)	92.9 (91.5, 94)	3.9 (1.3, 8.7)	99.7 (99.3, 99.9)	12.7

NPV = negative predictive value; PPV = positive predictive value. Sensitivity, specificity, PPV, and NPV are presented as percentage (95% confidence interval).

and were delivered before 34 weeks, half of those with placental abruption that required emergency delivery, and 80% of fetal deaths. In contrast with the high sensitivity for the preceding serious adverse outcomes, the overall sensitivity for preeclampsia was about 40% and about 20% for delivery of infants with birth weights less than the tenth percentile.

Sensitivity for preeclampsia and delivery of small infants at the severe end of the spectrum were similar to those achieved in other two-stage programs. Thus, in a study of 1326 women at 19 to 21 weeks' and 24 weeks' gestation, Harrington et al[4] reported that abnormal results on uterine artery Doppler were present in 3.9% of pregnant women, including 81.2% of those who developed preeclampsia before 34 weeks and 57.6% of those who delivered infants with birth weights below the tenth percentile before 34 weeks.

The main features of our study that distinguish it from previous ones are the use of color Doppler as the primary technique and relatively late gestation at screening. Most previous studies used continuous-wave Doppler to obtain waveforms from the uterine arteries without viewing them.[8,9] With color Doppler imaging, the precise location of uterine arteries as they cross the external iliac arteries is first identified and waveforms are obtained under direct vision with pulsed-wave Doppler. Gestation of 23 weeks was selected because previous studies that examined uterine

Table 4. Screening Characteristics for Bilateral Notches, Irrespective of Mean Pulsatility Index Above 1.45

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Relative risk
Preeclampsia (n = 65)	32.3 (21.2, 45.1)	96.7 (95.8, 97.5)	27.3 (17.7, 38.6)	97.4 (96.5, 98.1)	10.4
Preeclampsia, delivery before 34 wk (n = 10)	80 (44.4, 97.5)	96 (95, 96.9)	10.4 (4.5, 19.2)	99.9 (99.5, 100)	87.3
Birth weight below tenth percentile (n = 143)	13.3 (8.2, 20)	96.4 (95.4, 97.3)	24.7 (15.6, 35.8)	92.6 (91.3, 93.8)	3.3
Birth weight below third percentile (n = 52)	22.6 (12.5, 36.8)	96 (95, 96.9)	15.6 (8, 24.7)	97.5 (96.8, 98.3)	6.4
Birth weight below tenth percentile, delivery before 34 wk (n = 10)	50 (18.7, 81.3)	95.9 (94.8, 96.8)	6.5 (2.1, 14.5)	99.7 (99.3, 99.9)	21.8
Birth weight below third percentile, delivery before 34 wk (n = 9)	55.6 (21.2, 86.3)	95.9 (94.8, 96.8)	6.5 (2.1, 14.5)	99.8 (99.4, 99.9)	27.3
Fetal death (n = 6)	83.3 (35.9, 99.6)	95.8 (94.7, 96.7)	6.5 (2.1, 14.2)	99.9 (99.7, 100)	109
Placental abruption (n = 10)	50 (18.7, 81.3)	95.8 (94.7, 96.8)	6.5 (2.1, 14.3)	99.7 (99.3, 99.9)	21.8

NPV = negative predictive value; PPV = positive predictive value. Sensitivity, specificity, PPV, and NPV are presented as percentage (95% confidence interval).

Table 5. Screening Characteristics for Mean Pulsatility Index Above 1.45, Irrespective of Bilateral Notches

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Relative risk
Preeclampsia (n = 65)	35.3	96	25.8	97.5	10.3
	(23.9, 48.2)	(94.9, 96.9)	(16.7, 35.5)	(99.6, 98.2)	
Preeclampsia, delivery before 34 wk (n = 10)	80	95.4	8.1	99.8	75
	(44.4, 97.5)	(94.3, 96.4)	(4, 17.1)	(99.6, 100)	
Birth weight below tenth percentile (n = 143)	21	96.3	33.7	93.2	5
	(14.6, 28.6)	(95.2, 97.2)	(23.7, 44.1)	(9.9, 94.4)	
Birth weight below third percentile (n = 52)	66.6	95.1	4.5	99.9	37.5
	(52.9, 79.7)	(93.9, 96.1)	(21.6, 38.8)	(99.3, 100)	
Birth weight below tenth percentile, delivery before 34 wk (n = 10)	70	95.2	7.8	99.8	43.7
	(34.8, 93.3)	(94.1, 96.1)	(3.1, 15.2)	(99.5, 100)	
Birth weight below third percentile, delivery before 34 wk (n = 9)	77.8	95.3	7.9	99.9	65.6
	(40, 97.2)	(94.2, 96.3)	(3.2, 15.5)	(99.6, 100)	
Fetal death (n = 6)	66.6	95.1	5.6	99.9	37.5
	(22.3, 95.7)	(94.2, 96.3)	(1.2, 11)	(99.6, 100)	
Placental abruption (n = 10)	20	95	2.2	99.5	4.7
	(2.5, 55.6)	(93.9, 96)	(0.3, 7.9)	(99.1, 99.8)	

NPV = negative predictive value; PPV = positive predictive value. Sensitivity, specificity, PPV, and NPV are presented as percentage (95% confidence interval).

arteries at earlier gestations reported high false-positive rates (the reason that two-stage programs were developed). In those previous studies, patients were examined with routine second-trimester fetal anomaly scans (18 to 20 weeks' gestation), and those with abnormal uterine artery waveforms underwent second-stage screening, typically at around 24 weeks. For example, in the study of Harrington et al,[4] the screen-positive rate (defined as bilateral notches or a unilateral notch and resistance index of more than 0.55) at 20 weeks was 17%; this rate decreased to 8.9% at 24 weeks.

One of the major criticisms of uterine artery Doppler screening studies has been the excessive reliance on subjective assessment of uterine artery waveforms for presence or absence of early diastolic notches. Our study used only color Doppler equipment, which might be associated with more reproducible measurements of impedance indices than is continuous-wave Doppler. For the same screen-positive rate, sensitivity of PI for preeclampsia and delivery of infants with birth weights less than the tenth percentile might be better than that of bilateral notches. Although some adverse outcomes

Table 6. Screening Characteristics for Mean Pulsatility Index Above 1.45 and Bilateral Notches

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Relative risk
Preeclampsia (n = 65)	23	98.6	39.4	97	13.5
	(13.5, 35.2)	(97.9, 99.1)	(23.4, 55.4)	(96.2, 97.8)	
Preeclampsia, delivery before 34 wk (n = 10)	70	98.2	18.4	99.8	105
	(34.8, 93.3)	(97.5, 98.8)	(7.7, 34.3)	(99.5, 100)	
Birth weight below tenth percentile (n = 143)	11.8	98.7	44.7	92.6	6.1
	(7.1, 18.4)	(98, 99.2)	(28.6, 61.7)	(91.3, 93.9)	
Birth weight below third percentile (n = 52)	22.6	98.4	31.5	97.6	13.2
	(12.5, 36.8)	(97.7, 99)	(17, 47.6)	(96.8, 98.3)	
Birth weight below tenth percentile, delivery before 34 wk (n = 10)	50	98.1	13.1	99.7	45.2
	(18.7, 81.3)	(97.4, 98.7)	(4.4, 28.1)	(99.3, 100)	
Birth weight below third percentile, delivery before 34 wk (n = 9)	55.6	98.2	13.9	99.8	59.8
	(21.2, 86.3)	(97.5, 98.8)		(99.4, 99.9)	
Fetal death (n = 6)	66.6	98	10.5	99.8	90.5
	(22.3, 95.7)	(97.2, 98.6)	(2.9, 24.2)	(99.6, 100)	
Placental abruption (n = 10)	20	97.9	5.2	99.5	11.3
	(2.5, 55.6)	(97.1, 98.5)	(0.6, 17.3)	(99.1, 99.8)	

NPV = negative predictive value; PPV = positive predictive value. Sensitivity, specificity, PPV, and NPV are presented as percentage (95% confidence interval).

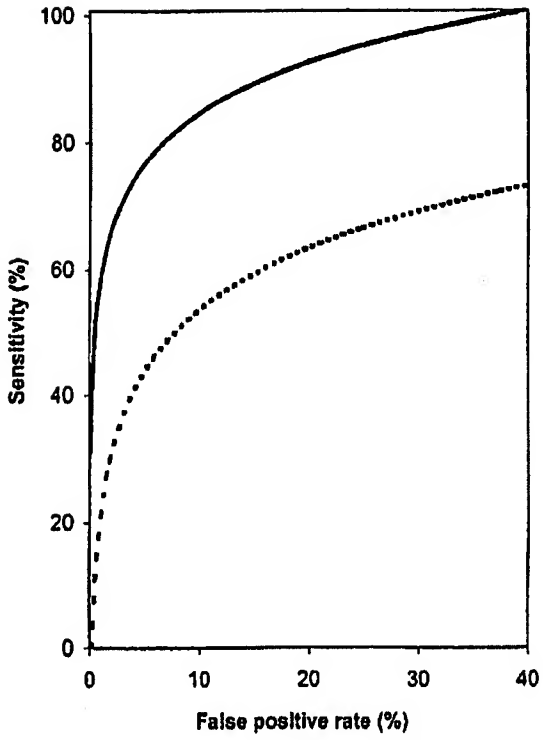

Figure 1. Receiver-operating curves of mean pulsatility index for preeclampsia. Curves of best fit are shown for the total group (*dashed line*) and for women who delivered before 34 weeks' gestation (*solid line*).

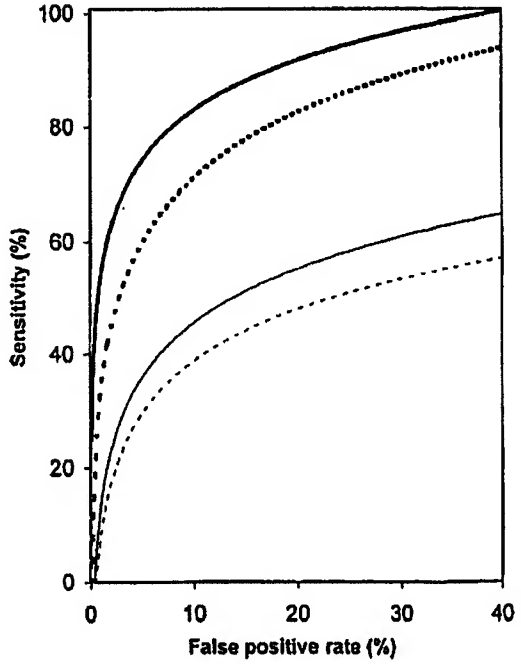

Figure 2. Receiver-operating curves of mean pulsatility index for delivery of a small-for-gestational-age infant. Curves of best fit are shown for birth weight less than the tenth percentile (*light dashed line*) and less than the third percentile (*light solid line*) for the total group and weight less than the tenth percentile (*dark dashed line*) and less than the third percentile among women with delivery before 34 weeks' gestation (*dark solid line*).

occurred in the group with bilateral notches and normal PIs, inclusion of bilateral notches in the definition of screen-positive group introduces an element of subjectivity in the program and nearly doubles the screen-positive rate but only marginally improves sensitivity.

Women at highest risk are those with bilateral notches and a high mean PI. They have a 40% chance of developing preeclampsia and 45% for delivering infants of birth weight less than the tenth percentile. Although they comprise only 2% of the screened population, the relative risks for adverse outcomes before 34 weeks and fetal death in that group range from 50 to 100, a clinical risk that merits close antenatal surveillance.

Another important finding of our study was the negative predictive value, which was more than 99% for adverse outcomes before 34 weeks, placental abruption, and fetal death. This finding suggests that uterine artery screening might be used to determine the appropriate level of antenatal care in specific women. Women with normal uterine artery Doppler results are unlikely to develop preeclampsia, FGR or placental abruption and therefore do not necessarily need antenatal follow-up that is as close as that required in women with abnormal uterine artery Doppler findings. The real value of this method of screening is that using a mean uterine artery PI above 1.45 predicts most women who will experience severe preterm consequences of impaired placentation.

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the prediction of preeclampsia and fetal growth retardation. Obstet Gynecol 1994;83:378–86.
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Address reprints requests to:
Christoph Lees, MD
Harris Birthright Research Centre for Fetal Medicine
King's College Hospital, Denmark Hill
London SE5 9RS
United Kingdom
E-mail: cclees@compuserve.com

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EXHIBIT 6

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/553,462	05/05/2006	Makrina Savvidou	HO-P03236US0	8934

29053 7590 07/14/2011
FULBRIGHT & JAWORSKI L.L.P
2200 ROSS AVENUE
SUITE 2800
DALLAS, TX 75201-2784

EXAMINER	
SINGH, ANOOP KUMAR	
ART UNIT	**PAPER NUMBER**
1632	

NOTIFICATION DATE	DELIVERY MODE
07/14/2011	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

doipdocket@fulbright.com

Interview Summary	Application No.	Applicant(s)
	10/553,462	SAVVIDOU ET AL.
	Examiner	Art Unit
	ANOOP SINGH	1632

All participants (applicant, applicant's representative, PTO personnel):

(1) *ANOOP SINGH*.	(3)_____.
(2) *Melissa L. Sistrunk*.	(4)_____.

Date of Interview: *29 June 2011*.

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☐ No.
If Yes, brief description: _____.

Claim(s) discussed: _____.

Identification of prior art discussed: *Holden et al. Am J Obstet Gynecol. 1998; 178(3):551-6*.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *Applicants' representative was contacted to inform an inadvertent typographical error in the office action mailed on December 9, 2010. It was indicated that description on page 3, line 5 of the office should read figure 1B instead of figure 1A as evident from the teaching described in the earlier sentence that correctly cites the teaching are derived from figure 1B of Holden et al. Applicants' representative was also informed that AMDA level greater than 0.75 is derived from the teaching of Holden and previously explained in the office action mailed on September 22, 2009 (see page 9, line 4).*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

/Anoop Singh/ Primary Examiner, Art Unit 1632		

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

EXHIBIT 7

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/553,462	05/05/2006	Makrina Savvidou	HO-P03236US0	8934

29053 7590 09/22/2009
FULBRIGHT & JAWORSKI L.L.P
2200 ROSS AVENUE
SUITE 2800
DALLAS, TX 75201-2784

EXAMINER	
SINGH, ANOOP KUMAR	

ART UNIT	PAPER NUMBER
1632	

MAIL DATE	DELIVERY MODE
09/22/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/553,462	SAVVIDOU ET AL.	
	Examiner	Art Unit	
	ANOOP SINGH	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *21 May 2009*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,6-8 and 11* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1, 6-8, 11* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Applicants' amendment to the claims filed May 21, 2009 have been received and entered. Claim 1 has been amended, while claims 2-5, 9-10, 12-28 have been canceled. Claims 1, 4- 11 are pending.

Election/Restrictions

Applicant's election without traverse of claims 1-2 and 4-11 in the reply filed on August 31, 2007 was acknowledged.

Claims 1, 6-8 and 11 are under consideration.

Maintained -Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1, 6-8 and 11 remain rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of determining that a pregnant woman is at risk of developing pre-eclampsia or whether that her fetus is at risk of developing intrauterine growth restriction (IUGR), which method comprises: (a) measuring plasma concentration of asymmetric dimethylarginine (ADMA) in a pregnant woman at risk of developing pre-eclampsia or her fetus being at risk of developing IUGR at a stage of pregnancy from 23 to 25 weeks gestation; and (b) plasma ADMA level in said women greater than 1.5 microM/L indicates that the woman is at risk of developing pre-eclampsia or her fetus is at risk of developing IUGR, does not reasonably provide enablement for determining that a pregnant woman is at risk of developing pre-eclampsia by measuring ADMA at any other stage of pregnancy. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Applicants' arguments filed May 21, 2009 have been fully considered and are persuasive in parts. Applicants' cancellation of claims 4-5, 9 and 10 renders their rejections moot. Applicants' amendment of base claim 1 to recites measuring the level of ADMA in the plasma level, only in part obviates the grounds for rejection.

Applicants argue that measuring ADMA in a plasma sample taken from pregnant woman at stage of pregnancy from 4 to 25 weeks gestation would not require undue experimentation. Applicants assert that one of skilled in the art could measure ADMA levels and compare to the threshold level. Applicants assert that the specification provides sufficient guidance to allow one of ordinary skill in the art to make and use the present invention without undue experimentation.

Such is not found persuasive, because instant analysis is based on the predictability of the risk of developing pre-eclampsia (PE) or IUGR is based on the presence of plasma ADMA level greater then 1.5 μmol/L at any stage of pregnancy. As stated before that applicants agree that pre-eclampsia is a multi factorial disease, which involves changes in, amongst others, a cardiovascular function, metabolic function and renal function (see Cooke et al Circulation. 2004 Apr 20;109(15):1813-8, Fard et al, Arterioscler Thromb Vasc Biol 2000; 20: 2039-2044 and Kielstein et al Am J Kidney Dis. 2005; 46: 186–202, Fang et al Hypertension 2006; 48: 724-729, all of the record). Thus, it is apparent that level of ADMA is influenced by different conditions and disorders and may not be specific risk marker for PE or IUGR in the first trimester. The guidance provided in the specification shows ADMA level greater than1.5 microM/L at 23 to 25 weeks of gestation indicates risk of developing pre-eclampsia or risk of developing IUGR in pregnant women.

It should be noted that instant claims embrace a method of determining that a pregnant woman is at risk of developing pre-eclampsia or that her fetus is at risk of developing IUGR by (i) measuring asymmetric dimethylarginine (ADMA) levels in "a" pregnant woman of varying gestational age (4-25 week of gestation) and (ii) determining that the women is at risk of developing PE or IUGR of the level of ADMA in the plasma is greater than 1.5 micro mol/L. With respect to applicants' argument that one could measure ADMA levels and compare to the threshold level, it is noted that as recited method of claim 1 step 1 recite measuring ADMA in any pregnant woman and does not require comparing to any control to arrive at any threshold

number as argued by the applicants. Further, the specification fails to disclose a threshold level of ADMA as a preclampsia risk indicator in pregnancy in first trimester. The specification teaches women with bilateral notches had significantly higher levels of ADMA compared to the women with normal uterine artery Doppler waveforms (2.4 μmol/L vs. 0.81 μmol/L respectively, Figure 2, page 21) which is at least three time of normal pregnancy level. With respect to applicants' argument that the data demonstrates that a plasma ADMA level measurement of greater than 1.5 micro mol/L can be used determine that a woman is at risk of developing IUGR or pre-eclampsia, it is noted that the specification discloses that women who subsequently developed pre-eclampsia had significantly higher levels (2.21-3.21μmol/L) of ADMA (Table 3), however, specification also teaches that women in absence of notches with raised concentrations of ADMA did not develop pre-eclampsia (see table 3). It is noted that only women with notches and a high ADMA developed pre-eclampsia. In the instant case, there is no evidence on record that establishes nexus between higher ADMA level in plasma of a pregnant woman at a first trimester stage of pregnancy from 4 to 15 weeks gestation. In fact, Ellis et al (Acta Obstet Gynecol Scand. 2001 Jul;80(7):602-8, IDS) report no convincing correlations between ADMA and clinical parameters (see page 606, col. 1, para. 1). In view of these comments, ADMA plasma levels cannot be used to determine risk of preclampsia in first trimester pregnancies. Furthermore, Contrary to the limitation of claim 7, Ellis et al teach that the ADMA/SDMA quotient is significantly lower in subjects with severe preeclampsia than in controls, reflecting a marked elevation of SDMA. These results are also contrary to the teaching of Pettersson et al (Acta Obstet Gynecol Scand 1998; 77: 808–813). The unpredictability of establishing nexus between first trimester uterine artery resistance and maternal serum concentration of ADMA is further evidenced by a recent report that states "[n]o significant difference was found in maternal serum ADMA between pregnancies with first trimester high resistance uterine artery blood flow and control" (abstract) (Prefumo et al Ultrasound Obstet Gynecol, 2008, 31, 153-157) (emphasis added). It is emphasized that several years after filing of this report Prefumo concludes that a long longitudinal study would be required to examine whether concentration of maternal ADMA in the first trimester co relates with the onset of the pre-eclampsia (se page 156, col. 1, last para.) MPEP 2164.05(a) also states "If individuals of skill in the art state that a particular invention is

not possible years after the filing date, that would be evidence that the disclosed invention was not possible at the time of filing and should be considered. In *In re Wright, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513-14 (Fed. Cir. 1993)*. Therefore, it is evident from the teaching of the cited references filed before, and after filing of this application show measuring ADMA level at different stage of pregnancy, without any comparison step of threshold value in base claims would not be reliable risk marker for PE or IUGR. It should be firther noted that the unpredictability of a particular art area may alone provide reasonable doubt as to the accuracy of the broad statement made in support of enablement of claims. See *Ex parte Singh*, 17 USPQ2d 1714 (BPAI 1991). It is also well established in case law that the specification must teach those of skill in the art how to make and how to use the invention as broadly claimed. In re Goodman, 29 USPQ2d at 2013 (Fed. Cir. 1994), citing *In re Vaeck*, 20 USPQ2d at 1445 (Fed. Cir. 1991). Given the lack of guidance provided by the specification it would have required undue experimentation for one of skill in the art to make and use the invention as claimed without a reasonable expectation of success. It is apparent that underlying condition and gestation age could greatly effect the level of plasma ADMA level. Given such differences in the level of ADMA at varying gestation age of a preganat woman as embraced by the claims, particularly when taken with the lack of guidance provided by the specification, it would require undue experimenattaion to make and use the inevntion without resonable expectation of sucess.

It is emphasized that the claims are directed to measuring ADMA levels in plasma of a pregnant women that is limited by determining that women is at risk of developing IUGR or PE if the level of ADMA is greater than 1.5 micro mol/L. Examiner had previously indicated that the state of the art generally recognized that the reference used for comparison with the test level of the AMDA level may vary, depending on aspect of the invention being practiced. These values are subjective to sample population, other variables (age, gender, hormonal status, ethnicity, disease state), assay system and are subjective to different interpretation by different artisans (see López -Jaramillo (J Hypertens. 2005; 23(6): 1121-9 and references therein, art of record). Given that levels of ADMA may vary depending on values that are subjective to sample population such as age, gender, hormonal status, ethnicity, disease state, it is thus unpredictable as to how one might use any reference marker profile comprising ADMA identified in a plasma in the analysis of a biomarker profile obtained from any other pregnant women with underlying

disease that influence ADMA level (kidney disease, hypertension). The specification fails to provide an enabling disclosure for the claimed invention because the specification fails to provide sufficient guidance as to how an artisan of skill would have practiced the claimed method in any pregnant women of any ethnicity suffering from multiple chronic disorders to predict the risk of developing PE or risk of fetus developing IUGR if ADMA level is greater then 1.5 micro mol/L (see the discussion before). An artisan would have to carry out extensive experimentation to make and use the invention, and such experimentation would have been undue because art of predicting that a pregnant women is at risk of developing PE by measuring the level of ADMA in any tissue or fluid without reasonable expectation of success.

New-Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1 and 7 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Boger (WO 2002/14873, 2/21/2002, IDS), Holden et al (Am J Obstet Gynecol. 1998; 178(3):551-6, art of record).

Claims are directed to method of determining that a pregnant woman is at risk of developing pre-eclampsia (PE) or that her fetus is at risk of developing intrauterine growth restriction (IUGR), which method comprises: (a) measuring asymmetric dimethylarginine (ADMA) in a pregnant woman at a stage of pregnancy from 4 to 25 weeks gestation; and (b) determining that the woman is at risk of developing pre-eclampsia or her fetus is at risk of developing IUGR if the level of ADMA is greater than 1.5 micromol/L in the woman. Instant rejection is applied to the extent claim reads on measuring if the ADMA level is greater than 1.17 micromol/L in the pregnant woman to suggest subject is at risk of developing PEWith respect to claim 1, Boger et al teach a method of detecting the risk of developing a disease including pre-eclampsia that is associated with NO metabolism by (a) measuring the level of

ADMA and SDMA (see claims 1 and 9). Boger et al also disclose that preeclampsia is a disease of the NO metabolism leads to constriction of arteries which induces high blood pressure in the mother and poses a risk to the unborn child due to reduced placental perfusion (see page 2) With respect to claim 7 and 8, Boger et al contemplate measuring the ration of ADMA to SDMA in the plasma of the patient (see claim 14, 20 and 21). It is also disclosed that subject suffering from chronic condition (CHF, example 4) show ADMA concentration of 4.1 μM/L as compared to 1.0 μM/L in normal subject. Boger teach a method of measuring ADMA in a subject to determine the risk of developing chronic condition if ADMA level is high and also contemplated to determine ADMA level in other conditions that increase high blood pressure such as PE, but Boger differed from claimed invention by not disclosing measuring ADMA level in pregnant women at a stage of pregnancy from 4 to 25 weeks gestation.

The deficiency of Boger is cured by Holden who teaches measuring plasma ADMA level in 145 pregnant women that included pregnancy of all stages (including second trimester). It is noted that Holden et al also determined the level of ADMA which is at least 0.52 μmol/L to 1.17 μmol/L depending upon stage of pregnancy. This would meet the breadth of the claim measuring pregnancy at different stage of pregnancy (4-25 weeks) that is embraced by the teaching of Holden (see page 553, Figure 1 B). It is noted that Holden et al conclude that during later stage of pregnancy circulating concentrations increase and, when pregnancy is complicated by preeclampsia. It should be noted that Holden et al teach that mean plasma ADMA level in PE subject was 1.17±0.42 (1.59-0.75μM/L) as compared to control subject. The range taught by Holden encompasses 1.5 uM/L as claimed. Thus, method of Holden is primarily directed to study the role for ADMA in the changes in blood pressure seen in preeclampsia pregnancy (see abstract and page 555, col. 1, para. 4).

It would have been obvious for one of ordinary skill in the art at the time of invention to modify the method of Boger by measuring the ADMA level in the pregnant women at varying stage of pregnancy from 4-25 weeks gestation using the known method disclosed by Holden. It would have been prima facie obvious to one of ordinary skill in the art to combine the known methods of Boger and Holden to measure the ADMA level in a pregnant women at a stage of pregnancy from 4-25 and determine the level of ADMA to predict the risk of developing

preeclampsia particularly since both generally embraced the potential of measuring ADMA level to determine the risk of developing pre-eclampsia. One who would have practiced the invention would have had reasonable expectation of success since Boger and Holden both taught method to measure ADMA level in the plasma of subject to determine if the subject is at risk of developing PE, while combining the teaching Boger and Holden would have resulted in a determining the level of ADMA that is greater then 1.17 μM/L to establish risk of developing pre-eclampsia.

Therefore, the claimed invention would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention.

"The art rejection may be overcome if applicant amended the base claim to include additional method step of carrying Doppler flow form analysis of the uterine arteries to determine pregnant woman with bilateral notches and than measuring asymmetric dimethylarginine (ADMA) in a pregnant woman at a stage of pregnancy from 23 to 25 weeks gestation; and (b)-determining that the woman is at risk of developing pre-eclampsia or her fetus is at risk of developing IUGR if the level of ADMA is at least 3 times greater than the ADMA level in the normal pregnancy ."

Response to arguments

Applicants' arguments filed May 21, 2009 have been fully considered but are not persuasive. Applicants' cancellation of claims 4-5 and 9 renders their rejections moot. Applicants argue that Boger and Holden fail to disclose or suggest determining that a woman is at risk of developing pre-eclampsia or her fetus is at risk of developing IUGR if the level of ADMA in a plasma sample is greater than 1.5 μM/L, as required by claim 1.

Such is not persuasive; because Holden et al teach that a level of ADMA in a pregnant woman of 1.17±0.42 (1.59-0.75μM/L) is sufficient to indicate that woman is at risk of developing pre-eclampsia. In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986). Applicants have further engaged in selective reading of the teachings of Boger and Holden to

formulate the grounds for not teaching the invention. It should be noted that the ultimate goal of measuring plasma ADMA in patient is to determine if the patient is at risk of developing PE. As previously indicated, contrary to applicants' assertion prior art of Holden recognized that higher ADMA level (greater than 1.17±0.42 or a range 1.59-0.75 μM/L) in the plasma of a pregnant woman is indicative of PE. Further, it is noted that claimed method recite two method steps (1) measuring ADMA in a plasma sample taken from any pregnant woman at a stage of pregnancy from 4 to 25 weeks gestation, and determining that the woman is at risk of developing pre-eclampsia or her fetus is at risk of developing IUGR if the level of ADMA in the plasma sample is greater than 1.5 μM/L. In the instant case, it would have been *prima facie* obvious to one of ordinary skill in the art to combine the known methods of Boger and Holden to measure the ADMA level in a pregnant women at any stage of pregnancy from 4-25 and determine the level of ADMA of more than 1.17±0.42 μM/L to predict the risk of developing preeclampsia particularly since both generally embraced the potential of measuring ADMA level to determine the risk of developing pre-eclampsia. One of ordinary skill in the art after studying Holden would recognize that ADMA level of greater than 1.17±0.42 μM/L in the plasma of a pregnant woman is sufficient to predict that the subject is at risk of developing pre-eclampsia. Thus, the rejection of claims 1 and 7 is maintained for reasons of record and the foregoing commentary.

Conclusion

No claims allowed.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event,

however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ANOOP SINGH whose telephone number is (571)272-3306. The examiner can normally be reached on 9:00AM-5:30PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Peter Paras can be reached on (571) 272- 4517. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Deborah Crouch/
Primary Examiner, Art Unit 1632

Anoop Singh
AU 1632

XI. RELATED PROCEEDINGS APPENDIX

None